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STUDIES ON THE SUPRARENAL CORTEX

II. METABOLISM, CIRCULATION, AND BLOOD CONCENTRATION DURING SUPRARENAL INSUFFICIENCY IN THE DOG

By GEORGE A. HARROP, M.D., ALBERT WEINSTEIN, M.D., LOUIS J. SOFFER, M.D., and JOHN H. TRESCHER, M.D.

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The details of the operative preparation, care, and maintenance of our colony of suprarenalectomized dogs were given in the preceding paper (1). The effect of injections of suprarenal cortical extract upon intact, as well as upon suprarenalectomized animals was described, together with its power to revive the latter from a state of acute insufficiency. It was pointed out that much of the work which has been done upon suprarenal function, as revealed by the effects of bilateral removal of the glands, is open to the serious objection that it was necessary to make the studies on animals which could hardly have recovered from the anesthesia and the effects of the necessarily severe operative procedures. In many of the observations reported in the literature moribund animals have undoubtedly been employed. The present experiments were carried out on animals maintained after operation in an active healthy condition with adequate amounts of cortical extract. The body weight had returned to the level present at the time of the first operation, and in many instances the state of nutrition had even improved. The studies were usually carried out several weeks after complete healing of the operative wounds. The sole variable factor therefore was the cessation of the daily injections of the cortical extract. No anesthesia was used in any of the experimental procedures to be recorded in this paper. We have discontinued the use of ether in the operations for removal of the glands, as we find the narcosis produced by nembutal equally satisfactory and much safer.

We describe in this paper the effects of acute suprarenal insufficiency upon the respiratory metabolism and upon the circulation and concentration of the blood of bilaterally suprarenalectomized adult male dogs.

Respiratory Metabolism and the Respiratory Quotient in Suprarenal Insufficiency

The collections of gas samples for analyses of the expired air for respiratory metabolism studies and experiments upon the blood flow were made upon trained, fasting animals lying at rest, in a warm quiet darkened room for 1 hour prior to the determinations. We have considered the simplest and best criterion that the animal is in a basal resting condition to be the pulse rate, which, except in insufficiency, is rarely over 70 per minute. During severe insufficiency the animal at rest will sometimes have a pulse rate of double or treble that of the intact or extract-treated suprarenalectomized animal. During the most extreme phases, changes in rhythm occur, so that idioventricular rhythm may lower the pulse to

TABLE I

Basal Oxygen Consumption of Suprarenalectomized Dog Following Withdrawal of Injections of Cortical Extract

Dog F-2-1, brown male terrier. Feb. 2, 1932, right suprarenalectomy. Feb. 29, 1932, left suprarenalectomy. Received injections of cortical extract twice daily until Apr. 16. None given thereafter.

Date	Weight	Temperature (rectal)	Pulse per min.	Respirations per min.	Respiratory volume per min.	Oxygen consumption per min.	Respiratory quotient	Room temperature	Comments
1932	*kg.*	*°F.*			*liters*	*cc.*		*°C.*	
Apr. 15	7.4	101.6	64	18	1.49	49.5	0.73	26.0	
									Extract injections stopped Apr. 16
Apr. 18	7.3	100.6	112	14	1.44	49.5	0.72	25.5	Without extract injections 48 hrs.
Apr. 20	7.0	99.7	68	20	1.64	40.0	0.69	25.2	Spastic gait. No food for 48 hrs. Non-protein nitrogen 80 mg. per 100 cc.
Apr. 21	6.8	96.0	38	18	2.01	39.0	0.70	26.0	Too deeply in relapse to be revived. Non-protein nitrogen. 155 mg. per 100 cc. Died 3 hrs. later

40 (Table I). The gas mask used was devised by Dr. H. F. Pierce (2). It is readily constructed and may be worn by the animal for periods of 2 hours or more without restlessness. It fits the muzzle snugly with a minimum of dead space. A set of these masks was made from various sizes of motorcycle or automobile inner tubing. The mask and respiratory valve (Lovén) were adjusted on the animal before the rest period was begun. 15 to 20 minute collections of the expired air were always made, in duplicate, often in triplicate, one period immediately following the other, after basal conditions were secured. It is important, as a precaution, to make sure that the bladder has been recently emptied before commencing the rest period. Certain animals could not be trained, without unusual difficulty, and these were discarded when a training period of a week proved insufficient. The collections were made with the usual precautions into 50 liter Douglas bags, a mixed sample was taken over mercury for gas analysis, and the total gas volume was then measured through a calibrated 3 liter wet meter. While

TABLE II

Basal Oxygen Consumption of Suprarenalectomized Dog Following Withdrawal of Injections of Cortical Extract

Dog P-2, male, mongrel terrier. Suprarenalectomized during Feb., 1931.

Date	Weight	Temperature (rectal)	Pulse per min.	Respirations per min.	Respiratory volume per min.	Oxygen consumption per min.	Respiratory quotient	Blood non-protein nitrogen	Comments
1931	*kg.*	*°F.*			*liters*	*cc.*		*mg. per 100 cc.*	
Apr. 7	11.0	100.5	76	12	2.64	56.1	0.73	56	Normal and active
Extract injections stopped Apr. 11									
Apr. 13	10.6	101.4	72	12	2.82	55.3	0.73	84	Most of food refused
Apr. 14	10.5	100.0	106	13	3.35	50.6	0.73	140	No food. Swaying gait
Apr. 15	10.0	98.4	108	12	3.12	Lost	Lost	170	Swaying. Vomiting
Large amounts of extract with intraperitoneal 5 per cent glucose 150 cc. No saline given									
Apr. 16	9.7	97.8	106	12	2.91	48.5	0.69	200	Drinks water and voids
Large amounts of extract with intraperitoneal 5 per cent glucose 150 cc. No saline given									
Apr. 17	9.7	99.2	108	12	2.68	47.8	0.69	170	Diuresis 500 cc.
During the next 4 days extract and glucose given in large amounts. No food eaten during this period									
Apr. 18	9.1								
Apr. 20	8.8								
Apr. 21	8.6	98.4	108	12	2.82	43.7	0.73	204	Severe insufficiency
Died during the night of Apr. 21–22									

TABLE III

Basal Oxygen Consumption and Respiratory Quotient of a Suprarenalectomized Dog Maintained over an Extended Period

Dog F-2-9, Mar. 28, 1932, right suprarenalectomy. Apr. 15, 1932, left suprarenalectomy, Dr. Penick. Daily injections of cortical extract thereafter except as noted in the table.

Date	Weight	Temperature (rectal)	Pulse per min.	Respirations per min.	Respiratory volume per min.	Oxygen consumption per min.	Respiratory quotient	Room temperature	Comments
1932	*kg.*	*°F.*			*liters*	*cc.*		*°F.*	
Mar. 17	8.4	102.0	76	14	1.43	52.3	0.76	24.0	
Mar. 18	8.0	102.0	64	14	1.48	54.6	0.70	26.0	
									12 cc. (240 dog units) cortical extract injected Mar. 22 and Mar. 23
Mar. 24	7.6	101.7	70	16	1.42	53.3	0.71	24.5	
									Right suprarenalectomy Mar. 28
Apr. 1	7.7	101.8	76	10	1.36	54.7		24.5	4th day after operation
Apr. 14	7.4	101.6	64	18	1.70	50.0	0.72	24.5	14th day after operation
									Left suprarenalectomy Apr. 15. Thereafter extract injected daily
Apr. 19	7.2	102.1	100	16	2.00	47.8	0.76	28.5	4th day after second operation
Apr. 21	7.1	101.1	68	16		50.0	0.72	25.5	6th day after second operation
									Extract injections stopped Apr. 23
Apr. 25	7.2	100.7	110	18	2.13	48.0	0.71	23.5	
Apr. 27	7.0	100.0	56	16	2.21	43.0	0.73	24.0	Spastic gait. Non-protein nitrogen 83 mg. per 100 cc. Had refused food 36 hrs.
Apr. 28 a.m.	6.8	98.2	44 (120)	16	3.10	40.9	0.69	25.0	Marked insufficiency. Cannot stand. Given extract and saline subcutaneously after test
Apr. 28 p.m.		100.6	46 (120)	18	4.05	43.0	0.74	28.0	Experiments done 6 hours later. Pulse shifts abruptly from 46 to 120
Apr. 29	6.7	102.8	98	16	2.90	41.2	0.69	28.0	Condition improved but no food taken. Non-protein nitrogen 66 mg. per 100 cc.

Apr. 30	6.8	101.2	120	13	2.14	37.4	0.73	24.5	Ate fair meal (155 gm. meat). Non-protein nitrogen 44 mg. per 100 cc. Relapse May 2 due to insufficient extract. Weight 6.3 kg. Non-protein nitrogen 128 mg. per 100 cc. Intraperitoneal saline and 13 cc. extract
May 3	6.6	101.4	76	11	2.01	35.8		28.0	Improved but very weak. Ate 300 gm. meat
May 5	6.8	100.5	68	12	2.11	42.0	0.75	27.0	
May 6	6.6	100.0	66	12	2.01	41.2	0.74	24.8	Much improved. Eating well
May 9	6.8	100.2	70	10	2.08	43.9	0.74	23.0	
May 11	6.7	100.7	90	10	2.16	46.2	0.75	24.0	
Nov. 4	7.0	101.0	90	7	1.47	41.0	0.73	26.0	No experimentation during the summer
Nov. 10	7.1	100.4	76	9	1.20	40.0	0.73	26.5	
Nov. 17	6.7	99.8	70	13	1.75	36.5	0.74	24.0	Extract injections stopped Nov. 11. Non-protein nitrogen 95 mg. per 100 cc.
Nov. 18	6.6	98.9	60	13	1.75	34.5	0.72	24.0	Well marked insufficiency. No food for 36 hrs. Non-protein nitrogen 115 mg. per 100 cc. Extract injections resumed with intraperitoneal saline
Nov. 30	7.1	100.3	60	8	0.92	39.0	0.68	26.0	Condition good. Eating well

Animal died in insufficiency following withdrawal of extract, Feb. 11, 1933 most of the gas analyses were made by one of us (G. A. H.) the gas collections and analyses were made for several experiments by Dr. Edward M. Bridge and Miss E. M. Bridges, whose assistance is acknowledged.

The essential findings indicated in typical experiments in Tables I–III are: (1) the cortical extract even in large amounts, adrenalin-free, has no immediate influence on the oxygen consumption of the normal resting dog; (2) the basal oxygen consumption is not changed by removal of one gland, or by removal of both, if adequate amounts of cortical extract are supplied so that the body weight and state of nutrition are maintained; (3) when insufficiency is produced in the bilaterally suprarenalectomized dog by withdrawal of injections of the extract, a drop in the basal oxygen consumption occurs to 20–25 per cent below the original level when the animal shows severe symptoms. The lowering of the basal metabolism occurs only after a considerable interval following the withdrawal of extract, and is more or less coincidental with the fall in temperature. The changes in the respiratory quotient are not large, and such lowering as occurs is probably due to the anorexia and fasting characteristic of severe insufficiency. All of the animals have been on a meat (fat-protein) diet during the period of experimentation, unless otherwise noted. The respiratory volume frequently, but not invariably, rises during the stage of insufficiency. There is generally a well marked lowering of the plasma bicarbonate content. The basal oxygen consumption in Dog F-2-9, while it was receiving adequate amounts of extract, dropped about 20 per cent in the course of 8 months (Table III). At the same time the body weight dropped 12–15 per cent although the animal appeared in excellent condition and in a good nutritional state. We have previously described the absence of hyperplasia in the thyroid glands of these animals, which are maintained without suprarenal glands over long periods, as well as the frequent calcification of the colloid which is seen on histological section.

Blood Flow

Determinations of the circulatory minute volume were made by the Fick (3) method on animals trained for respiratory metabolism studies. After the oxygen consumption was determined under basal conditions as outlined above, samples of mixed venous and arterial blood were immediately obtained, and analyzed in the

TABLE IV

Sodium Chloride and Nitrogen Balance and Excretion of Urea, Creatin, and Creatinine in Suprarenalectomized Dog Following Withdrawal of Injections of Cortical Extract

Dog F-7, black haired male terrier. Oct. 14, 1931, right suprarenalectomy. Nov. 11, 1931, left suprarenalectomy. Fluid given by stomach tube to make up 200 cc. intake during the period the animal was not given extract. No salt given except in the food, which contained 0.38 per cent of chlorides calculated as NaCl. Urine chlorides calculated as NaCl.

Date	Length of period	Weight	Fluid intake per 24 hrs.	Food per 24 hrs.	Urine output per 24 hrs.	Total N_2 intake per 24 hrs.	Urine N_2 per 24 hrs.	N_2 balance per day	Urea N_2 per 24 hrs.	Creatin N per 24 hrs.	Creatinine N per 24 hrs.	Blood non-protein nitrogen	Sodium chloride intake per 24 hrs.	Sodium chloride output per 24 hrs.	Sodium chloride balance per day
1931	*days*	*kg.*	*cc.*	*gm.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*mg. per 100 cc.*	*gm.*	*gm.*	*gm.*
Nov. 28–Dec. 5	7	9.3	320	390	124	5.7	5.6	+0.1	4.9	0.27	0.27	32	1.48	1.46	—
Dec. 5–Dec. 12	7	9.2	280	375	123	5.5	5.8	−0.3	4.8	0.36	0.28	38	1.43	1.43	—
		Extract injections stopped Dec. 12. None given thereafter until death													
Dec. 12–Dec. 15	3	9.0	200	180	128	2.6	4.7	−2.1	4.0	0.77	0.25	70	0.69	1.60	−0.91
Dec. 15–Dec. 16	1	8.7	200	None	160	—	3.5	−3.5	2.9	1.34	0.23	100	—	0.71	−0.71
		Dog voided only a few cubic centimeters on Dec. 16 and died during the night of Dec. 16–17													

Van Slyke constant volume blood gas apparatus, the collections being made and the procedure carried out essentially as described by Barcroft (4), and later adapted to dogs by Marshall (5). Oxygen capacity was determined from oxygen content after aeration of a portion of the arterial blood in the blood gas pipette in the usual manner (Sendroy (6)). A typical experiment is shown in Table VI.

TABLE V

Urinary Volume and Chloride Excretion of Suprarenalectomized Dog Following Withdrawal of Injections of Cortical Extract

Dog 1-3, male collie. Nov. 21, 1930, right suprarenalectomy. Feb. 2, 1931, left suprarenalectomy (Dr. Widenhorn).

Date	Length of period	Weight	Blood nitrogen		Urine volume per day	Chlorides given as sodium chloride		Oxygen capacity	Comments
			Non-protein	Urea					
1931	*days*	*kg.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*cc.*	*gm.*		*vol. per cent*	
May 3–7	4	15.4	36	17	232	1.4	Extract	20.3	
May 7–8	1				176	1.3	Extract	20.4	
May 8–9	1		42	22	195	2.5	No extract		
May 9–11	2	15.6	56	33	208	3.2	No extract	21.0	
May 11–13	2	15.5	52	32	318	2.6	No extract		
May 13–15	2	15.4	66	45	248	1.1	No extract		
May 15–18	3	14.7	140	94	280	0.7	Extract	24.4	Severe insufficiency. Low blood pressure. No food for 48 hrs.
May 18–19	1	15.1	122	68	320	0.5	Extract		
May 19–21	2	14.7	41	20	767	0.6	Extract		
May 21–25	4	14.5	34	11	490	1.2	Extract	20.5	Complete recovery

No diminution in urinary output even with maximal blood nitrogen concentration and hemoglobin concentration. Increased excretion of chlorides following extract withdrawal later falling when body stores were depleted. Nitrogen and urea excretion shown on Chart 1, *J. Exp. Med.*, 1933, 67, 312.

Experiments on the circulatory minute volume confirm the assumption made in our previous paper that a marked drop in the cardiac output and in the output per beat occurs in severe suprarenal insufficiency. They show also the unsaturation of the venous blood as well as the increased arterial oxygen content, due to the increased hemoglobin concentration. These factors combine to produce an increased arterial and venous oxygen difference. The percentage oxygen saturation of the arterial blood is normal throughout. The increased pulse rate in these animals, which usually appears as insufficiency becomes severe, does not result, therefore, in an increased cardiac output, but in a greatly diminished output per beat. These experiments indicate clearly the disorganization and failure of the circulation which develops during the progress of the insufficiency.

TABLE VI

Circulatory Minute Volume in Suprarenalectomized Dog Following Withdrawal of Injections of Cortical Extract

Dog F-7-0, Dec. 13, 1932, right suprarenalectomy. Dec. 22, 1932, left suprarenalectomy. Received injections of cortical extract twice daily. Room temperature maintained at 24°C.

Date	Weight	Temperature (rectal)	Pulse per min.	Respirations per min.	Respiratory volume per min.	Oxygen consumption per min.	Respiratory quotient	Circulatory minute volume	Blood oxygen analyses			
									Capacity	Arterial content	Venous content	Arterial-venous oxygen difference
1933	*kg.*	*°F.*			*liters*	*cc.*		*liters*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
Jan. 23	9.1	100.0	68	18	1.27	49.2	0.69	0.83	20.4	19.8	14.8	5.0
Extract injections stopped Jan. 24												
Jan. 27	8.7	100.3	76	18	1.23	52.0	0.73	0.64	22.7	22.4	14.3	8.1
In severe insufficiency Jan. 30. No food for 24 hrs. Spastic gait. Non-protein nitrogen 132 mg. per 100 cc.												
Jan. 30	7.8	96.8	118	20	1.18	40.3	0.68	0.38	25.5	25.0	14.1	10.9
Revived at once with injections of extract, intraperitoneal saline, and glucose. In excellent condition again on Feb. 13												
Feb. 13	8.7	101.0	70	17	1.24	50.9	0.71	0.66	23.3	22.5	15.9	6.6

Blood Pressure

The blood pressure during the various phases of suprarenal insufficiency was determined by means of a rectangular rubber cuff, measuring 5 by 15 cm., wrapped about the upper part of the thigh and attached to a mercury manometer. The rubber cuff was bound together with adjustable tapes, so that the differences in the diameter of the leg could be compensated for exactly, and the cuff could be closely applied and kept firmly in position. This method permits of repeated studies without pain or disturbance, and is satisfactory in larger animals with narrow thin extremities. The sound made by the pulse wave as it breaks through at the systolic level is readily heard through a stethoscope, provided with a small bell, which is placed over the artery as it emerges below the cuff.

Blood pressure changes in the resting animal occur only after injections of extract have been stopped for some time, and are usually coincidental with the appearance of the other evidences of circulatory failure (Table VII) when concentration of the blood is well advanced.

TABLE VII

Changes in Blood Pressure in the Suprarenalectomized Dog during the Course of Insufficiency Produced by Withdrawal of Injections of Extract

Dog 8-4, spotted white collie. Jan. 26, 1932, right suprarenalectomy. Feb. 2, 1932, left suprarenalectomy.

Date	Weight	Temperature (rectal)	Blood pressure	Oxygen capacity	Non-protein nitrogen	Comments
1933	*kg.*	*°F.*	*mm.*	*vol. per cent*	*mg. per 100 cc.*	
Feb. 8	9.9	101.6	120/70	17.1	34	Condition excellent
Feb. 10	9.8		130/70			
Feb. 14	10.0					Extract injections stopped
Feb. 18	9.5	100.0	135/75		50	4th day without extract. Active, eating well
Feb. 20	9.2	100.4	130/85	18.2	90	
Feb. 21 a.m.	9.0	100.0	106/60		96	Very ill. Weak. Vomited
p.m.	8.9	98.0	55/10	20.4	108	Severe insufficiency. Can rise with difficulty. Given large amounts of extract and intravenous and intraperitoneal salt solution
Feb. 22	8.7	100.7	136/80			Greatly improved. Can walk without stagger
Feb. 23	8.5	100.6	130/80			Improved further
Mar. 8	9.6	102.0	130/80	16.9	40	Condition excellent

Blood Concentration

Studies of erythrocyte counts in systemic and peripheral blood, of packed red cell volume, and of blood oxygen capacity were made in samples of blood withdrawn by femoral artery puncture, which were prevented from clotting by the use of purified heparin.[1] Studies were made, daily, during the period following

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[1] The heparin was repurified according to the method of Dr. W. H. Howell from material obtained from Hynson, Westcott, and Dunning, Baltimore. In 2 per

TABLE VIII

Hemoconcentration Following Extract Withdrawal from Suprarenalectomized Dogs (Femoral Artery Blood)

Date	Weight	Oxygen capacity	Hematocrit	Plasma volume	R.B.C.	Non-protein nitrogen	Total protein	A/G ratio	Comments
Dog 64, male									
1932	*kg.*	*vol. per cent*	*per cent*	*per cent*	*millions*	*mg. per 100 cc.*	*per cent*		
Mar. 8	7.2	10.5	26.5	72.0	4.24	34	6.41	49/51	Taken off extract a.m., Mar. 8
Mar. 9	7.2	10.8	27.5	70.0	4.42	46			
Mar. 10	7.1	11.0	28.5	70.0	3.60	40			
Mar. 11		11.6	29.5	68.5	3.84	48			
Mar. 12	7.1	14.4	36.5	61.5	5.07	55			Food intake diminished, but dog is active
Mar. 13	7.0	14.5	38.5	60.0	5.42	84			Food intake 50 gm.
Mar. 14	6.8	15.4	37.0	61.0	5.50	116			Temperature 99.8°. Vomited. Very quiet
Mar. 15	6.6	16.5	41.0	57.5	6.09	184	7.17	43/57	Temperature 97°. Marked stagger gait and weakness, deep insufficiency. Peripheral red blood cells 6.17 millions
Dog 80, male									
1932	*kg.*	*vol. per cent*	*per cent*	*per cent*	*millions*	*mg. per 100 cc.*	*per cent*		
Mar. 6	7.6	13.7	33.1	65.4	4.70	42	5.81	49/51	Taken off extract a.m., Mar. 6
Mar. 7	7.5	14.05	31.0	67.5	4.92	52			
Mar. 8	7.3	14.4	35.0	64.0	4.84	50			
Mar. 9	7.1	15.6	37.0	62.0	5.63	80	6.25	49/51	Temperature 99.8°. Food intake less than 50 gm., but dog is active
Mar. 10	7.0	14.9	35.2	63.8	5.70	90			Temperature 98.3°
Mar. 11	6.8	16.4	39.0	60.5	5.96	144	6.90	49/51	Temperature 98°. Extremely quiet, diarrhea, definite insufficiency. Peripheral red blood cells 5.55 millions

withdrawal of extract, and the results were correlated with the coincidental rise in blood non-protein nitrogen, plasma protein concentration, and the loss in body weight (Table VIII).

The observations show that hemoconcentration usually begins within 24 hours after withdrawal of extract, and that the loss of plasma volume, as judged from the volume of packed erythrocytes may be from 10–35 per cent in severe insufficiency. There is no increased concentration of erythrocytes in the peripheral blood (ear) as contrasted with the venous blood, such as has been described in shock due to trauma or hemorrhage (7). Transudation of fluid into the tissues does not occur, characteristically, in shock due to suprarenal insufficiency, unless it takes place when the animal is moribund. As we shall indicate, fluid passes in the opposite direction, namely from tissues to capillaries. An increased erythrocyte concentration therefore is not to be expected in the peripheral vessels.

Water and Salt Excretion during Insufficiency

Data showing the increased excretion of sodium chloride following cessation of injections of extract in suprarenalectomized dogs are presented in Tables IV and V. They are selected from experiments in which a carefully measured food intake of known composition was given. These data also indicate that the volume output of urine is well maintained following withdrawal of extract, and that anuria occurs only during the terminal circulatory collapse, when the blood pressure also falls rapidly.

DISCUSSION

The experiments which are described show clearly that the process involved in suprarenal cortical insufficiency, due solely to withdrawal of injections of the hormone, is essentially a condition of "shock," associated with progressive loss of plasma volume, lowered general metabolism and blood flow, and, terminally, lowered body temperature and blood pressure.[2] We have shown previously (1) that it is

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cent solution it is neutral in reaction, and very effective in preventing clotting. It is a highly useful anticoagulant for chemical studies, and in the purified form referred to above can be used in minute amounts.

[2] The extreme muscular weakness of these animals in insufficiency is in part due to the condition of "shock," but, together with the curious characteristic spastic

characteristically accompanied by a rise in plasma non-protein nitrogen and a fall in chlorides. The approximate time of the appearance of these various phenomena, following the withdrawal of the injections of hormone, and the extent of their change from the original condition is shown in a composite chart of a typical group of six suprarenalectomized animals (Chart 1).

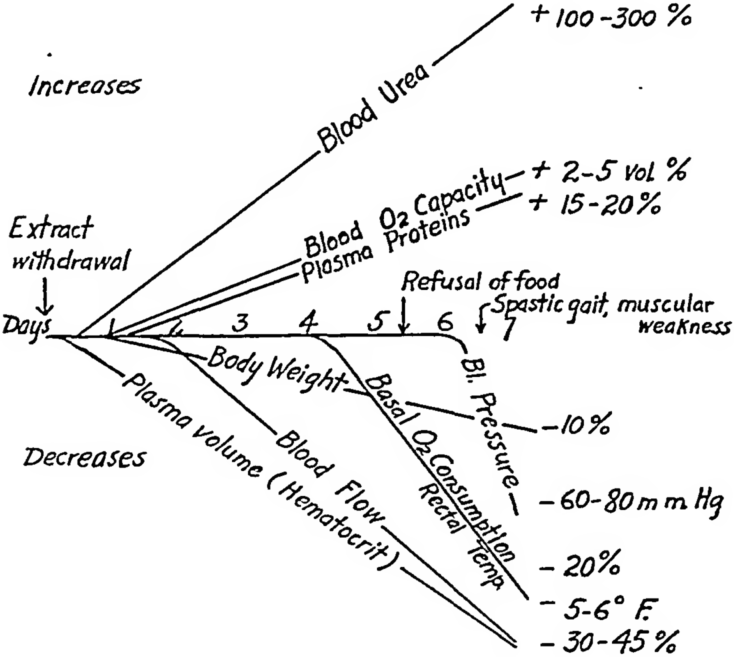

CHART 1. Characteristic changes in metabolism, circulation, and blood concentration in a group of suprarenalectomized animals following withdrawal of extract.

The condition of "shock," the concentration of the blood, and the circulatory collapse have been noted following removal of the suprarenal glands repeatedly, but most observers have been unable to de-

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weaving gait, may also be due to the disturbance of the relative concentrations of the basic ions, potassium, magnesium, and sodium, in the tissues and body fluids. We expect to report on muscle and other tissue analyses having a bearing on this question.

cide how far these are due to the major surgical procedure involved in removal of the suprarenal glands, as well as the anesthesia, and how far they are due to the loss of the cortex itself. It is only when the suprarenalectomized animal is restored to a normal nutritive condition with healed incisions, and when the sole variable consists in withholding the daily injections of the cortical extract, that the changes can be ascribed to the withdrawal of extract, and to this alone. When the animal is afterwards restored to its former condition by resumption of injection of hormone the cycle is completed.

The mechanism by which this condition of "shock" and eventual death in suprarenal insufficiency is brought about next attracts attention. Loss of water from the blood plasma may occur in various ways. Where increased capillary permeability exists, it may diffuse into the extravascular tissues, a mechanism which has been described in traumatic shock and in the shock produced by the injection of histamine. On the other hand it may be lost through the gastrointestinal tract as in cholera or by emesis, as in high intestinal obstruction. Finally the loss may occur through the kidneys associated with a loss of electrolytes, a mechanism which in part explains the blood concentration and the condition of shock which may occur in diabetic coma, or in certain cases of uremia.

The possibility of loss of plasma water into the tissues by increased capillary permeability has been suggested as the explanation of the shock of suprarenal insufficiency in a recent preliminary communication by Swingle (8). The theory, at present generally accepted, as to the mechanism which maintains the balance of fluid exchange between capillaries and extravascular spaces, relates it to the opposing forces of colloid osmotic pressure and capillary blood pressure (or the difference between these pressures and their homologous components in the tissues). The changes in both of these forces will act to draw fluids into the capillaries in suprarenal insufficiency unless the wall itself is damaged. The concentration of plasma proteins increases during suprarenal insufficiency, as we have indicated above, but without much change in the albumin-globulin ratio. This should tend to increase the colloid osmotic pressure of the plasma and should hold fluid more firmly within the vessels. At the same time the blood pressure falls, thus reducing the force which tends to drive fluid out of the blood plasma into the tissue spaces. The general condition of the tissues at autopsy is of interest. The viscera are dry and the skin and subcutaneous tissues are markedly inelastic, as though devoid of water. There is no increase in the erythrocyte count in the peripheral tissues over that in the systemic blood. Hence there is no alteration in the distribution of the red blood cells such as is said to occur in traumatic shock. A loss of 10 per cent or more of the body weight during the days following withdrawal of extract is very common. Vomiting and diarrhea cannot explain this loss of blood volume or of body weight, because these symptoms only occur very late in insufficiency after the blood concentration has become marked.

We turn therefore to the remaining alternative, that dehydration, following cessation of the injections of cortical hormone, is due to loss of water through the urine. The amount of fluid excretion necessary to produce a concentration of the blood of the extent found in suprarenal insufficiency is quite compatible with the urinary volume observed. If we assume the total plasma volume of Dog F-2-9 to be 500 cc., a reduction of 40 per cent would require an excess excretion of but 200 cc. in the course of 8–9 days. Much more than that may actually occur. Balanced experiments indicate quite clearly that there is no reduction in urinary volume in insufficiency until within a few hours of death.

Hemoconcentration due to loss of fluid from the body produces the circulatory collapse seen in suprarenal insufficiency and will adequately explain the observed train of symptoms: loss of weight, lowered body temperature, and the gastrointestinal symptoms including anorexia, diarrhea, and vomiting. It is therefore unnecessary to postulate any hypothetical "toxin" which is neutralized in the intact cortex or by its hormone, as the cause of the symptoms and of death. The "shock" of suprarenal insufficiency has analogies with that produced in collapse due to exposure to high temperatures in which great loss of salt occurs through the sweat.

SUMMARY

1. The basal oxygen consumption, respiratory quotient, blood flow, blood pressure, and the changes in concentration of the arterial blood were determined in suprarenalectomized dogs (*a*) when receiving in-

jections of cortical extract (Swingle-Pfiffner), (*b*) during suprarenal insufficiency induced by withdrawal of the injections, and (*c*) during the period of recovery brought about by resumption of extract injections.

2. Reasons are advanced for the view that the hemoconcentration which occurs is probably due to loss of fluid through the kidneys, rather than by increased capillary permeability and loss into the tissues, or by loss through the gastrointestinal tract.

3. The loss of fluid from the plasma and tissues will explain the symptoms which follow withdrawal of injections of the hormone, as well as the fatal outcome. The assumption that the suprarenal cortex or its hormone has a detoxifying action upon some product of metabolism is thus rendered unnecessary.

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STUDIES ON THE SUPRARENAL CORTEX

III. Plasma Electrolytes and Electrolyte Excretion during Suprarenal Insufficiency in the Dog

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In the preceding papers (1, 2), we have described the phenomena produced by the cessation of injections of suprarenal cortical extract in the suprarenalectomized adult dog, which has been maintained in a normal state of nutrition, with well healed incisions, for a period of several weeks after suprarenalectomy. We have pointed out that death in such an animal, if injections of the hormone are not resumed, is due to a condition of shock produced by loss of body fluid. The observed sequence of events is: hemoconcentration, which becomes more and more marked; progressive loss of body weight; anorexia; lowered body temperature and basal oxygen consumption; muscular weakness; vomiting and diarrhea; and ultimate failure of the circulation, as indicated by diminished blood flow and fall in blood pressure. These symptoms are associated with a progressive rise in blood non-protein nitrogen (urea) concentration, a drop in plasma chlorides, and, as we have recently shown (1), a fall in plasma total base. The present communication is concerned with an analysis of the factors producing this hemoconcentration and of the train of phenomena which then follows it.[1]

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[1] We wish to acknowledge the assistance of Dr. Mary Buell in organizing the chemical procedures involved in the manufacture of the cortical extract and in the selection and setting up of analytical methods used in the studies herein reported.

We are indebted to Miss Margaret Strauss who has made lactic acid, inorganic phosphorus, and calcium estimations on a series of animals before and during insufficiency.

We acknowledge the important assistance of Dr. Oliver Kamm, of Parke, It is well known that changes in the distribution of body fluids are often accompanied by disturbances in the normal electrolyte structure of the blood plasma. Loeb and his coworkers have made an important contribution to the elucidation of this problem in experimental suprarenalectomy (19). They have shown that the sodium concentration of the blood decreases both in suprarenalectomized dogs and cats and in patients suffering from Addison's disease. By balance studies they have shown that a striking loss of sodium occurs due to loss of sodium in the urine and that the behavior of the chloride ion follows that of the sodium, but the loss is not equivalent. They discuss the possibility that the suprarenal glands have a regulatory effect upon the sodium metabolism and upon renal function. They have previously shown that similar changes occur in the blood of patients with Addison's disease which may be materially modified by the administration of sodium chloride. We have, therefore, determined the concentrations of the plasma electrolytes during the cycle of events which takes place following the cessation of injections of extract as the animal goes into suprarenal insufficiency, and during the progress of recovery when injections of extract are resumed.

Procedure

Samples of (femoral) arterial blood were taken for urea estimations and study of the electrolyte pattern. They were obtained with an oiled syringe by direct puncture of the vessel. We have found that adequate protection from the air is secured by delivery of the blood through a bent glass tube into 15 cc. centrifuge tubes, provided with a drop of purified heparin (6 per cent solution), and a flat glass bead. Each centrifuge tube is filled to the top and immediately capped with a rubber stopper, such as is used for vaccine bottles, through which a needle is inserted, the tip just emerging at the lower end. This permits the escape of air bubbles, followed by any excess of blood. The tubes are vigorously turned end for end for a minute or two to secure complete mixing of the blood and heparin. On centrifuging, a clear non-hemolyzed plasma invariably results. Carbon dioxide determinations are first made by removing the stopper, and inserting the measuring pipette to remove the sample from the lower half of the supernatant plasma, without disturbing the red cell mass below. The duplicate is taken from a second centrifuge tube, and the plasma is then immediately separated from the cells.

Davis and Company, who has generously supplied us for the past 2 years with the beef suprarenal glands from which we prepare our cortical extract.

Because the operation for suprarenalectomy is technically difficult in larger animals and the maintenance extract requirement is proportionally greater, we have used male dogs exclusively, weighing between 7 and 10 kilos. Owing to the rather large quantities of blood required for repeated analyses, we have restricted our studies, in one group of experiments, to estimations of total base, bicarbonates, chlorides, sodium, and urea (or non-protein nitrogen) (Chart 1, Tables V, VII). Studies of calcium, magnesium, and potassium, inorganic phosphates, lactic acid, and total proteins, with the partition of the ratio of albumin to globulin, were usually done in separate groups of experiments (Tables I and II).[2]

The results show that during the course of suprarenal insufficiency induced by cessation of injections of extract, a fall occurs in the plasma total base concentration, the extent depending in great measure on the degree of insufficiency into which the animal is allowed to lapse. This is associated with a considerable rise in the concentration of magnesium and potassium, but without marked increase in that of calcium. Since the total concentration of plasma base fell, and since that of the other constituents rose, the results of our earlier experiments pointed to a decided drop in the plasma sodium concentration. This

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[2] *Methods of Analysis.*—

Estimations of *total base* were made by the method of Stadie and Ross (*J. Biol. Chem.*, 1922, 51, 55), with certain modifications; of *sodium* by that of Butler and Tuthill (*J. Biol. Chem.*, 1931, 93, 171); of *potassium* (plasma only) by that of Taylor (*J. Biol. Chem.*, 1930, 87, 27); *calcium* and *magnesium* by that of Kramer and Tisdall (*J. Biol. Chem.*, 1921, 47, 475); *carbon dioxide* by that of Van Slyke and Neill (*J. Biol. Chem.*, 1924, 61, 523); *oxygen capacity* by that of Sendroy (*J. Biol. Chem.*, 1931, 91, 307); *inorganic phosphate* by that of Fiske and Subbarow (*J. Biol. Chem.*, 1925, 66, 375); *non-protein nitrogen* by direct nesslerization (Wong, S. Y., *J. Biol. Chem.*, 1923, 55, 431); *urea* by decomposition with urease and direct nesslerization, using gum ghatti as a stabilizing colloid (Folin, O., *J. Biol. Chem.*, 1929, 81, 231); *plasma chlorides* by the method of Van Slyke and Sendroy, (*J. Biol. Chem.*, 1923, 58, 523); *lactic acid* by that of Friedman *et al.* (*J. Biol. Chem.*, 1927, 73, 335, and *J. Biol. Chem.*, 1929, 82, 23); *plasma proteins* by difference of the results of total nitrogen and non-protein nitrogen concentration estimated by the Kjeldahl method; and the *albumin-globulin ratio* by Howe's Kjeldahl method (*J. Biol. Chem.*, 1921, 49, 109).

Standard methods were employed for estimations of urine constituents: *total nitrogen* by macro Kjeldahl digestion and titration; *creatine* and *creatinine* by Folin's method (*J. Biol. Chem.*, 1914, 17, 475); *ammonia* by aeration into acid and titration of the excess (Folin, O., *J. Biol. Chem.*, 1910, 8, 497).

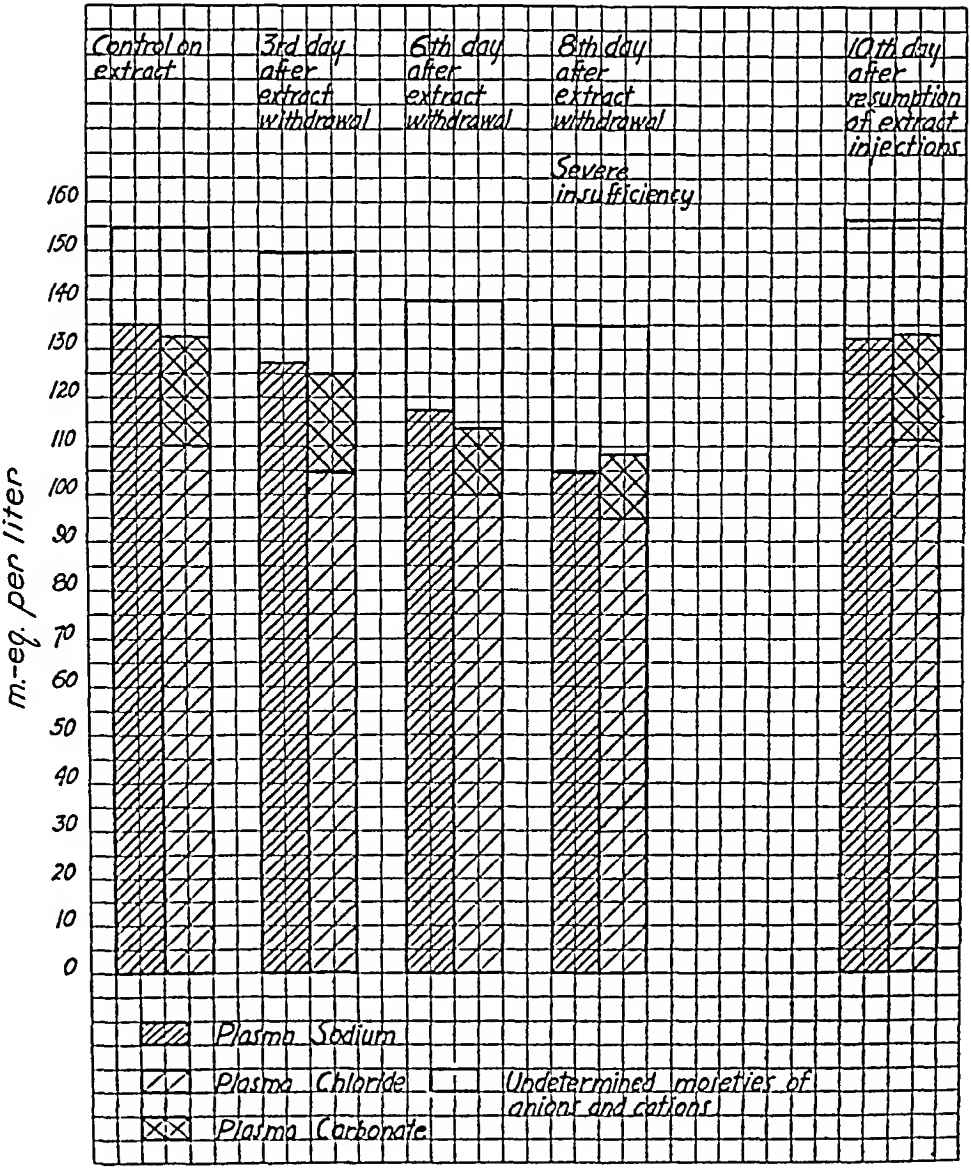

Chart 1. Plasma electrolyte pattern during the course of suprarenal insufficiency following withdrawal of extract from the suprarenalectomized dog.

we have confirmed directly. The change in concentration may amount to 20 m.-eq. per liter or even more.[3] Since the concentration

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[3] The fall in plasma sodium, which is accompanied by an appreciable rise in plasma potassium concentration, produces a marked alteration in the ratio nor-

TABLE I

Plasma Cations and Total Base Values in Normal Dogs and in Dogs with Suprarenal Insufficiency

Normal animals							Suprarenal insufficiency						
Dog No.	Plasma calcium per liter	Plasma magnesium per liter	Plasma potassium per liter	Plasma total base per liter	Plasma sodium* per liter	Plasma sodium/potassium	Dog No.	Plasma calcium per liter	Plasma magnesium per liter	Plasma potassium per liter	Plasma total base per liter	Plasma sodium* per liter	Plasma sodium/potassium
	m.-eq.	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*			*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	
6-0	6.1	1.5	4.7	157.0	144.7	30.6	6-0†	7.5	2.3	4.9	147.8	131.1	26.6
7-0	7.2	1.3	4.7	150.6	137.4	29.2	7-0	7.9	2.4	9.8	139.0	118.9	12.1
1-9	6.2	1.5	6.2	154.4	140.5	22.6	1-9	5.8	1.7	10.2	150.4	132.7	13.0
5-6	6.2	1.4	6.2	152.6	138.8	22.2	5-6	8.2	3.8	13.9	146.8	120.9	8.7
6-8		1.5	5.5	152.2	138.2‡	25.1	6-7	7.7	4.1	10.0	134.6	112.8	11.3

* Sodium values obtained by subtraction. Total base − (Ca + Mg + K).

† Mild insufficiency only.

‡ Calcium lacking. Sodium estimated for assumed normal calcium of 7.0 m.-eq.

TABLE II

Blood Lactic Acid and Plasma Inorganic Phosphate Concentration in the Suprarenalectomized Dog

Dog W-1-3.

Date	Lactic acid	Blood non-protein nitrogen	Plasma inorganic phosphate	Clinical condition
1931	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
Feb. 25	8.8	60		Rather marked symptoms
Feb. 26	13.1	84		Severe insufficiency
Mar. 2	7.6	40		Active. Condition excellent
Mar. 9	7.3	44	5.1	Condition good
Mar. 13	9.0	50	5.7	Condition good
Mar. 16	14.6	82	8.2	Insufficiency. Staggering gait
		45		Extract given
Mar. 17	11.3	65	7.1	Improved
Mar. 23	6.5	45		Active, alert
Mar. 30	6.3	43	4.4	Active, eating well
Apr. 24	7.6	56	5.2	Condition good
May 7	6.0	36	4.6	Condition good
May 18	8.7	140	10.1	11th day after extract withdrawal severe insufficiency

of total base is believed to delimit the total concentration of plasma electrolytes, this latter quantity must be greatly lowered, and will account for the diminished plasma conductivity long ago noted by Stewart (7). There is also a progressive and steady fall in the concentration of bicarbonates and chlorides, the sum of the change being roughly equal to that of the sodium. The bicarbonate concentration drops before that of the chlorides, but the fall in the latter in severe insufficiency exceeds that of the bicarbonate. This is associated in the later stages with a rise in the concentration of inorganic phosphates. We have not made sulfate analyses, but the experiments of Swingle and Wenner indicate a similar rise in sulfate concentration (4). The increases in the concentrations of plasma proteins and of calcium may be explained as due to the plasma concentration itself. There is no increase in the concentration of lactic acid during the progress of insufficiency; no animals were studied, however, during convulsions (Table II).

The rate of recovery of the normal relations of the several plasma constituents following the readministration of potent cortical extract depends (*a*) on the degree of the insufficiency produced before measures for resuscitation are undertaken, and (*b*) on the extent to which accessory measures, namely the use of fluids, sodium chloride, and glucose, are employed in addition to the injections of cortical extract. Where large amounts of extract are injected the recovery is rapid, unless the animal has been allowed to become moribund, in which case it cannot be restored to the original condition (1).

Studies of the inorganic constituents of the blood and plasma of normal and suprarenalectomized cats under ether anesthesia, by the use of modern methods, were made by Baumann and Kurland (5) in 1926. They observed a drop in the proportion of plasma in the blood (65 per cent control, 57 per cent in insufficiency), and an increase in plasma solids. A fall of 15 per cent occurred in the sodium concentration and 9 per cent in that of chlorides. They further observed an increase in the concentration of potassium and magnesium, but little change in that

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mally existing between these ions in the plasma (Table I), and, it may be anticipated, in the tissue fluids as well. We are studying the possibility that this change may in part account for the weakness and spasticity of the voluntary muscles during the later stages of insufficiency, as well as for the curious disturbances of the cardiac rhythm (1) and the eventual heart failure.

of plasma calcium. They found no noteworthy change in the inorganic or total acid-soluble phosphates. Lucas (6) as well as Stewart and Rogoff (7) found evidence of similar blood concentration in dogs, and the former reported the decrease in blood chlorides in the dog. Baumann and Kurland pointed out the probable importance, but unknown significance, of the changes in the ratio of sodium to potassium in the blood of the suprarenalectomized cat. Hastings and Compere (8), following the changes in the blood serum of dogs after suprarenalectomy, found a progressive fall in the bicarbonate content, a marked and consistent fall in lactic acid, and an increased concentration of serum proteins and calcium, the latter effects being explained by the hemoconcentration. They stressed the marked increase in serum potassium, which they found attained at death the very high value of 20 m.-eq. per liter. No changes were observed in the creatine or creatinine concentration until a terminal rise took place commencing 24 hours before death.

We have previously indicated (2) that the loss of plasma volume during the progress of the insufficiency can be explained by increased permeability of the capillaries, and hence loss into the tissues; by loss through the gastrointestinal tract, due to vomiting or diarrhea; or, finally, by excretion of fluid through the urine. We have advanced reasons in our previous paper to show why it cannot be satisfactorily explained by drainage from the capillaries into the extravascular space, a process which is said to occur in traumatic shock, and following injections of histamine. We have also pointed out that it cannot be lost by vomiting, as in intestinal obstruction, nor by diarrhea, as in the condition of hemoconcentration and shock seen in cholera, since these effects do not occur until very late during insufficiency.[4]

We turn therefore to the consideration of loss of body fluids through the kidneys. We have previously pointed out that the volume of urine is well maintained until the blood pressure falls, late during the course of the insufficiency. During the days immediately following extract withdrawal the output is usually increased. Since a loss of body

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[4] During the final stages of suprarenal insufficiency, when the animal is in deep "shock," it is possible that both loss into the tissues by increased capillary permeability, and by vomiting and diarrhea, may account for the disappearance of water as well as electrolytes from the plasma. Proof is entirely lacking that either of these routes constitutes an important channel for the loss of the water and electrolytes which initiate the hemoconcentration.

water is commonly accompanied by a loss of the electrolytes with which it is associated in the tissues (9), balance experiments with measurements of fluid and food intake, and urine and stool output, have been carried out to ascertain what fluid and electrolyte changes may occur.

The dogs have been maintained in a well lighted room heated with thermostatic control at 70–78°F. Metal wire metabolism cages were used. The sides and floor were washed down with distilled water at the beginning of the experiment and between the periods of urine collection. The wash water was analyzed for its constituents, and the values found added to the total excretion for the period. Stools were removed and analyzed separately. Fecal excretion of sodium and of chlorides cannot be neglected. By reason of the impossibility of exact separation into periods it constitutes an uncertain source of error. The dogs were catheterized at 9.30 a.m. at the beginning of each control period, and daily during the stage of extract withdrawal and recovery. The weight was determined on silk scales to 0.05 kilos, immediately after catheterization. After removal of the urine, the bladder was washed out with a measured volume of sterile distilled water, which was added to the specimen. The urine specimens were collected under toluol with precautions against evaporation, measured daily, and kept on ice. Rectal temperatures were taken and observation made of the blood pressure by the method previously described (2). The water intake was measured and corrected for evaporation by subtracting the loss from a similar container suspended beside the cage.

The animal under study was given its food at noon and trained to consume it within 5 hours, during the fore periods. Complete consumption of food during the period when extract is withdrawn is sometimes difficult to secure. The uneaten food was thus weighed back with a minimal loss from evaporation since it was exposed only during this 5 hour period. Where salt mixtures were given in addition, the material was enclosed in a bolus of the food which was placed at the back of the dog's mouth. The muzzle was held shut until the animal was induced to swallow. Half of the prescribed dose was given at noon and half at the end of 5 hours.

The food intake for the balance experiments was given in two forms: *(a)* a proprietary canned dog food, (cooked salted horse meat), the chloride and sodium content of which was uniform as checked by control analyses, and *(b)* ground raw lean beef muscle to which definite amounts of sodium chloride were added as described above. The material was passed twice through the electric grinder to insure thorough mixing, weighed out in oiled paper, and preserved by refrigeration in ventilated tins until needed. Aliquot portions were taken for analyses for water, nitrogen, sodium, and chlorides. The chloride and sodium content of the canned dog food, as well as its nitrogen content, proved uniform within 5 per cent. The fresh beef muscle contained variable amounts of fat and was somewhat more irregular in chloride and sodium. Both were low, however, in comparison to the extra sodium chloride administered, and hence this error was not great in proportion to the total intake.

We have been constantly engaged in making balance experiments for the past 2 years. The technique upon suprarenalectomized dogs is difficult and the results presented are selected from a group of successful experiments to show the typical changes.

During the control periods the animals were injected subcutaneously with cortical extract in divided doses, twice daily. The same batch of extract was used throughout the experiment. Our extract is now so standardized that its strength, as determined by assay, is fairly constant (10).

TABLE III

Effect of Salt-Free Diet in Accelerating Suprarenal Insufficiency after Extract Cessation

Dog 2-9. Right suprarenalectomy, Mar. 28, 1932. Left suprarenalectomy, Apr. 15, 1932. Death in insufficiency, Feb. 11, 1933.

Extract discontinued, Apr. 21	Insufficiency on 7th day	Salted diet
Extract discontinued, May 11	Insufficiency on 10th day	Salted diet
Extract discontinued, Nov. 9	Insufficiency on 9th day	Salted diet
Extract discontinued, Dec. 2	Insufficiency on 10th day	Salted diet
Salt-free diet from Feb. 1 (weight 8.1 kg.)		
Extract discontinued, Feb. 7	Insufficiency on 2nd day	Salt-free diet
Death on 4th day. Could not be revived		

It was observed early that the salt intake of a suprarenalectomized animal has an important bearing on its behavior after withdrawal of extract. Dog 2-9 illustrates this fact (Table III).

This animal was known among our laboratory group as a "10 day" dog, because under the usual dietary regime with ample salt intake, it regularly went into marked suprarenal insufficiency, on the 8th to the 10th day following cessation of injections of the extract. Such individual regularity of behavior after extract withdrawal, when conditions are uniform, is characteristic of the suprarenalectomized dog. When this animal was then given a salt-poor (lean muscle meat) diet for several days, abrupt stoppage of extract injections then produced symptoms of severe insufficiency in 48 hours, and death, in spite of all efforts to revive the animal, 36 hours later.

The salt content of the food also has a significant bearing on the extract requirement. Dog 7-0 illustrates this fact (Table IV).

TABLE IV

Dog 7-0. Right suprarenalectomy, Dec. 13, 1932. Left suprarenalectomy, Dec. 22, 1932. Removal of salt from the diet where the extract dosage is at the maintenance level produces insufficiency. Conversely extract is required even where the diet is adequate in sodium.

Date	Weight	Extract per day	Remarks	Plasma total base per liter	Plasma sodium per liter	Plasma chlorides per liter	Plasma non-protein nitrogen
1933	*kg.*	*cc.*		*m.-eq.*	*m.-eq.*	*m.-eq.*	*mg. per 100 cc.*
Feb. 11 to 17	9.0 9.9	2	Mixed diet. Dog in excellent condition, gains 0.9 kg. in 6 days. Temperature, 101°	152.8	143.1	111.2	37
Feb. 17 to 22	9.9 8.8	2	Lean raw beef muscle without added salt. Loss of 1.1 kg. in 5 days. Feb. 22, dog in insufficiency. Can hardly stand, will not eat. Temperature, 99.3°. Given 5 gm. NaCl—intravenous and intraperitoneal infusions	144.6	132.7	105.1	113
Feb. 23	8.5	2	Somewhat improved. Ate small amount of food. Salt given by mouth, intravenously and intraperitoneally				
Feb. 24	8.5	2	Ate very well				43
Mar. 3	9.2	2	Maintained on this dosage of extract in good condition until Mar. 17, gaining 0.9 kg. in weight on normal salted diet	154.1	143.2	111.2	37
Mar. 17 to 27	9.4	2	Lean raw beef muscle with 1.5 gm. added salt. The salt protects the animal although on the identical diet and extract dosage which previously threw him into insufficiency (Feb. 17 to 22)	153.3	140.9	108.9	38
Mar. 27 to 31	9.4 8.8	No extract	Extract removed Moderate insufficiency. Blood pressure not lowered	147.9	132.1	107.8	64

This animal was placed upon a low salt (whole chopped beef muscle) diet following the use of a well salted mixed diet, (mixed meat scraps, potato, bread, and vegetables from the hospital kitchen), on which it had been well maintained on an established level of extract dosage for weeks. After 4 days upon the low salt diet, this constant dose of extract from the same lot proved inadequate for maintenance, and it lapsed into insufficiency. The dog was restored to its normal condition by the usual measures and was then replaced on the same low salt diet (whole chopped beef muscle), but with the addition of 1.5 gm. of sodium chloride daily. On this regime the original dosage taken from the identical lot of extract maintained it in excellent condition.

TABLE V

Effect of Salt Content of Diet on Fall in Electrolyte Concentration and Production of Suprarenal Insufficiency after Cessation of Extract Injections. On Low Salt Diet the Symptoms of Insufficiency Appear Much Earlier and the Changes in the Plasma Electrolyte Pattern Are More Marked

Dog 8-4. Right suprarenalectomy Jan. 26, 1932. Weight 9.7 kilos. Left suprarenalectomy Feb. 2, 1932.

Date	Weight	Non-protein nitrogen	Plasma total base per liter	Plasma sodium per liter	Plasma chlorides per liter	Plasma bicarbonate per liter	Remarks
1933	*kg.*	*mg. per 100 cc.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	
Feb. 13	10.0	34	154.8	140.0	116.8	23.6	Mixed diet, well salted
	Extract injections stopped Feb. 14						
Feb. 17	9.9	50		137.1	112.8		
Feb. 19	9.3			131.0	106.0	17.2	
Feb. 21	8.9	96		128.2	103.0	14.2	Severe insufficiency. Temperature, 98°. Vomits, staggers. Blood pressure 55/0
Feb. 22	Revived with cortical extract and intravenous saline solution—8th day after cessation of extract						
Mar. 6	9.7	40		143.0	112.0	23.7	
Mar. 8	Extract injections stopped (weight 9.6 kg.)						Salt-free diet (ground lean meat) from Mar. 3, + 1.5 gm. NaCl
Mar. 11	8.8	100		131.5	102.6	15.8	Marked insufficiency
Mar. 13	8.4	160		121.9	95.6	13.6	Severe insufficiency. Appears almost moribund
Mar. 13	Revived with cortical extract and intravenous saline solution—5th day after cessation of extract						
Apr. 6	9.4	33		140.1	112.2	22.9	

The magnitude of the salt intake introduces a new variable into the assays of cortical extract upon the suprarenalectomized dog. Our previous work was done upon animals whose food intake was weighed daily. The salt intake was ample but it was not measured. It is

TABLE VI

Dog 2-9. Right suprarenalectomy Mar. 28, 1932. Left suprarenalectomy Apr. 15, 1932 (Penick). Balance experiment—canned cooked salted horse meat.

Day	Cortical extract per day	Weight	Urine volume per day	Nitrogen balance per day	Sodium balance per day	Chloride balance per day	Creatine per day	Creatinine per day	Total creatinine per day	Total phosphates per day	Blood non-protein nitrogen	Food intake per day	Clinical condition
	cc.	kg.	cc.	gm.	m.-eq.	m.-eq.	gm.	gm.	gm.	gm. P	mg. per 100 cc.	gm.	
1	5	7.0											
2	5												
3	5		Average per day										Control period
4	5		145	−0.1	−0.7	−0.3	0.12	0.25	0.37	0.16		300	
5	5												
6	5	7.1									34		
	Extract injections discontinued beginning Nov. 10, 1932												
7	0		218	−0.1	−27.9	−25.3	0.15	0.31	0.46	0.14		300	Condition excellent
8	0		244	+0.1	−35.6	−27.0	0.25	0.29	0.54	0.14		300	
9	0	7.0	247	0.0	−27.7	−22.0	0.24	0.28	0.52	0.14		300	
10	0		167	+0.5	−7.5	−4.1	0.22	0.23	0.45	0.15		300	
11	0	6.8	185	+0.5	−3.7	+1.0	0.19	0.25	0.44	0.13		300	
12	0		230	+0.1	−3.1	+1.0	0.22	0.27	0.49	0.16	90	300	Quite active. Eats food promptly
13	0		179	−0.4	−5.0	−2.5	0.23	0.23	0.46	0.12		200	
14	0	6.7	158	−0.8	−10.9	−6.1	0.16	0.21	0.37	0.11		95	
	Extract injections resumed Nov. 18, 1932												
15	15	6.6	290	−3.2	+5.0	+8.4	0.30	0.31	0.61	0.34	115	100	Very weak, but can stand. Temperature 98.4°.
16	10		202	−3.1	+1.5	+1.4	0.21	0.20	0.41	0.23		70	Much more active but ate very little
17	10	6.3	170	−0.7	+3.9	+7.7	0.28	0.31	0.59	0.27	45	300	
18	5		Average per day										
19	5		147	+0.1	+3.9	+2.9	0.15	0.28	0.43	0.19			
20	5	6.6									40	300	Condition excellent
21	5												
22	5		Average per day										
23	5		184	0.0	+7.1	+0.5	0.13	0.26	0.39	0.16		300	
24	5	6.8									27		

evident that the salt intake per kilo of body weight must be given close consideration. It is clear that the dosage may be reduced to a fraction of its original amount when a high salt diet is substituted for one low in salt content.

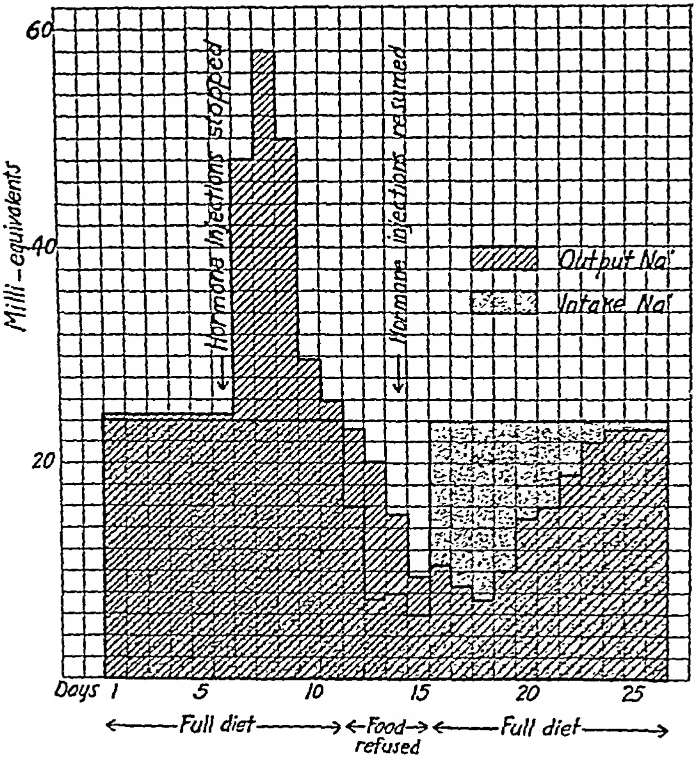

Chart 2. Sodium intake and output following cessation of cortical extract injections in a suprarenalectomized dog. The return to approximate balance on the 10th day is due to the exhaustion of the stores of extracellular water, and the fall in intake is due to refusal of food during the later stages of insufficiency. The sodium retention following resumption of the cortical extract injections about equals the loss during the earlier cessation period. Salted meat diet; no parental fluids given. Weight at beginning of experiment regained at its conclusion.

The results of these balance experiments (Tables VI, VII), indicate that after cessation of the injections of cortical extract in an animal on a measured diet and sodium chloride intake, an abrupt rise in the excretion of sodium, chloride, and water occurs. The sodium

TABLE VII

Dog 8-4. Right suprarenalectomy Jan. 26, 1932. Left suprarenalectomy Feb. 2, 1932. Balance experiment—raw lean beef muscle 450 gm. + 2.3 gm. sodium chloride daily.

Day	Cortical extract per day	Weight	Urine output per day	Fluid intake per day*	Fluid balance per day	Nitrogen balance per day	Phosphate urine per day	Sodium balance per day	Chloride balance per day	Plasma volume	Oxygen capacity	Plasma total base per liter	Plasma sodium per liter	Plasma bicarbonate per liter	Plasma chlorides per liter	Blood non-protein nitrogen	Plasma urea nitrogen	Plasma total protein	Albumin-globulin ratio	Food
	cc.	*kg.*	*cc.*	*cc.*	*cc.*	*gm.*	*gm. P*	*m.-eq.*	*m.-eq.*	*per cent*	*vol. per cent*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*m.-eq.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*gm. per 100 cc.*		*gm.*
1	2.5	9.2	Average per day																	450
2	2.5		458	670	+212	+0.7	0.66	+1.1	+4.6											450
3	2.5	9.2																		450
4	5.0									62.8	16.9	156.6	142.1	22.9	112.2	48	33	5.26	1.0	450
5	5.0		Average per day																	450
6	5.0		422	683	+261	+0.9	0.65	+3.8	+0.1											450
7	5.0	9.3																		450
		Extract injections discontinued																		
8	0	9.3	353	423	+70	+0.4	0.33	−26.3	−27.4				146.3	22.0	112.2		32			250
9	0	9.0	685	563	−122	+0.2	0.67	−61.0	−53.1											450
10	0	8.7	529	668	+139	−0.4	0.52	−12.3	−10.1				134.9	18.0	106.0	56	45			330
11	0	8.6	450	570	+120	+0.2	0.33	−3.2	+6.8											200
		Extract injections resumed																		
12	20.0	8.5	508	593	+75	−6.6	0.93	+24.3	+10.9	51.5	21.7	143.4	128.2	15.7	103.2	102	90	6.46	0.9	150
13	15.0	8.3	573	958	+385	0.0	0.78	+23.6	+23.4		20.3	144.6	134.2	21.0	105.2	40	23			450
14	8.7	8.6	484	678	+194	+0.6	0.77	+16.6	+13.9											450

15	4.4	8.8	Average per day							64.0	17.0	150.6	137.7	23.3	111.7		18			450
16	4.4	8.9	424	703	+279	+1.1	0.65	+8.2	+19.4											450
17	0.9	8.9										152.8	143.1	23.6	114.8		14	5.24	1.1	450
18	0.9		Average per day																	450
19	0.9	9.0	434	660	+226	+0.1	0.67	+0.4	+0.7											450

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* Fluid intake includes water content of food. The fluid balance does not take account of water loss through feces, expired air, or skin.

excretion is at its maximum during the following 48 to 72 hours. The excretion then falls with the drop in the concentration of these ions in the blood plasma, but the animal may remain on a negative balance even at the lowest plasma level until it is restored by injections of extract. Sodium is still excreted in appreciable amounts in the urine at a plasma sodium level of 106 m.-eq. per liter and chloride at a plasma chloride level as low as 85 m.-eq. per liter. The concentration of sodium in the urine increases following extract cessation. Notwithstanding the increased urinary volume, the concentration of this ion for the first 2 or 3 days may be nearly doubled.

The loss of sodium and of chloride through the urine in excess of the amounts ingested during the period of extract cessation is much greater than can be accounted for by the loss due to the fall in their plasma concentration, and it is evident that the excess must come from the tissues. Thus during the 48 hours following extract cessation (Dog. 8-4, Table VII, days 8 and 9), the excretion of urinary sodium in excess of that of the control period amounted approximately to 85 m.-eq. During the same period the fall in plasma sodium was 11.4 m.-eq. per liter. Even assuming the blood volume to be 1000 cc. and the plasma volume 600 cc., the loss from the plasma alone could account for but 6.8 m.-eq. of sodium (600/1000 × 11.4). The rest, over 90 per cent of the whole, or 78.2 m.-eq. must be withdrawn from the water depots outside of the blood, during this period. It is true that a certain absolute shrinkage occurs in the plasma volume, but it is small during these early stages. It is clear that in suprarenal insufficiency, as has been elsewhere observed, the changes in the concentrations of plasma electrolytes do not necessarily reflect, either in a qualitative or in a quantitative sense, the shifts in water and electrolytes which may be taking place in the depots of body water, or in the water and electrolyte balance of the body as a whole. It will be noted that the increased excretion of sodium takes place chiefly during the days immediately following extract cessation and that the excretion then falls off abruptly (Chart 2). We interpret this fall as due to the exhaustion of the stores of interstitial body fluid. Once depleted, further supplies of sodium are obtained from the intracellular fluids with greater difficulty and probably with more serious consequences to the organism. The increased sodium output in the urine, corrected for the amounts yielded by the blood plasma during this period, may well define the total volume of interstitial fluid so lost from the body.

During the recovery period, there is a positive balance with retention of sodium and chloride, a phase which may continue over several days after injections of extract are resumed. When the total loss of these electrolytes during the cessation period is compared with the retention during the recovery period it is found that these values approximate each other, provided the body weight returns to its original value. At the conclusion of the after period, the animal is again in sodium and chloride equilibrium and the plasma electrolyte pattern is restored approximately to its original form.

Immediately after the resumption of injections of cortical hormone, an increased excretion of urine is observed for a period of 24 to 72 hours. Simultaneously the urinary nitrogen is greatly augmented. During this time the body weight continues to fall although the animal may obviously be improving. Such a lag or even further loss of weight nearly always occurs before the body weight begins to return to its original level with the use of adequate injections of the cortical extract.

The sodium chloride intake in the food must be adjusted at a rather low level in order to demonstrate clearly the effects of removing the injections of cortical hormone. Where ample salt is supplied in the food to compensate for the loss through the kidneys, the body stores of sodium are not drained and no immediate pronounced effect is noted on the balance. On the other hand, for the purpose of demonstrating the loss of body salt after removal of the extract, the salt intake must not be too low, since in such circumstances a state of insufficiency (Table V, March 8 to 11) may appear abruptly, with vomiting and diarrhea, which ruin the observations.

It has been observed repeatedly during assay studies that animals which continue to eat can be maintained on lower amounts of extract for longer periods than those whose appetite is capricious. The effect may readily be explained as due to the lowered salt intake and consequent increased drain upon the endogenous salt stores of the animals whose food intake is low, and hence whose salt intake is greatly reduced.[5]

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[5] During the summer of 1932 we were unable to explain the high blood non-protein nitrogen values which we found in our suprarenalectomized dogs. For

Neither the nitrogen nor the phosphate excretion is materially increased during the period of extract cessation in spite of the loss of weight. On the contrary there is often a fall in excretion which becomes marked when food is refused. We have shown previously that during this phase a marked rise in blood urea concentration also takes place, notwithstanding the continued excretion of an equal or greater volume of urine. In the after period, following the injection of effective amounts of extract, there is an immediate increase in the urinary excretion of nitrogen, and the urea fraction is particularly affected. This is coincident with the return to normal of the blood urea level. As the concentration of blood urea is an accurate index of the concentration throughout the tissues of this freely diffusible substance, it is reasonable to suppose that the increased urinary excretion of urea is derived largely from this source. Coincident with it there is also an increased excretion of urinary phosphorus. This is interpreted as being derived from the protein destruction resulting from cellular disintegration during the period of insufficiency.

DISCUSSION

Loeb and his coworkers (19) have just published a paper upon the electrolyte balance in three dogs during an extended control period,

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weeks they were maintained clinically in an active healthy condition with blood non-protein (urea) nitrogen levels of 80 to 120 mg. per 100 cc. (65 to 100 mg. per 100 cc. urea nitrogen), a concentration which is ordinarily associated with severe insufficiency. Injections of large amounts of the cortical hormone produced relatively little effect upon this blood level. We were at first inclined to attribute it to some unknown substance which came through the various lipid extractions in the manufacture of our extract due to the fact that the glands were exposed to unusually hot weather at the abattoir before use. One animal, apparently in excellent condition, which also received 30 cc. daily of cod liver oil showed a constant elevation for several weeks up to 200 mg. per 100 cc. of non-protein nitrogen. It becomes evident, however, on reviewing the protocols during that period, that the diet of the animals consisted principally of boiled unsalted beef. Since the water in which it was cooked was discarded, much of the soluble sodium chloride was undoubtedly extracted and lost. A ration very low in salt content was thus given. The seasonal variation in the survival period following bilateral suprarenalectomy in various animal forms has been noted by many observers, as well as the fact that hibernating forms survive longer than those which are active. We have not had convincing evidence, however, that the extract requirement of our animals, maintained in healthy condition, is any greater in the warm summer months than it is during the winter.

followed by suprarenalectomy. The animals were then observed until death in suprarenal insufficiency. The magnitude of the disturbance in the electrolyte balance which we have found in the suprarenalectomized animal following cessation of injections of the cortical hormone, and during the subsequent repair of the process following resumption of its use, is quite comparable to that reported by these authors. It is evident that the effects on the electrolyte balance immediately following suprarenalectomy and those due to removal of injections of the cortical hormone are identical, in the dog. Loeb reaches the conclusion that the suprarenal glands have a regulatory effect upon sodium metabolism and upon renal function.

The reciprocal relations existing between the plasma concentrations of sodium and chlorides, and of urea, during both the period of extract cessation and that of recovery following its resumption, recall the theory, recently again revived by Blum and his coworkers (11), and by Hartman and Darrow (12), that urea nitrogen retention may be a compensation for the loss of electrolytes, in an effort to maintain osmotic equilibrium.[6] However doubtful such an explanation may be, the changes are so striking that the existence of some significant relationship seems probable.

It is evident that the retention of nitrogen during insufficiency cannot be due to a reduction of water available for urea excretion, nor can the accumulation be merely secondary to circulatory failure and fall in blood pressure (20). As pointed out above there is an actual increase in urinary volume following extract withdrawal, and the collapse of the circulation occurs only after the rise in blood non-protein nitrogen is marked. The evidence points clearly to a direct regulatory influence exerted by the hormone upon the renal excretion of the several substances under discussion.

The consequences of the loss of sodium, chlorides, and water through the kidneys in suprarenalectomized dogs, as a result of lack of the

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[6] The changes in the electrolyte pattern in suprarenal insufficiency do not affect the plasma pH (colorimetric method of Cullen and Sendroy (*J. Biol. Chem.*, 1922, 52, 501), except in the later stages, when a fall occurs which may be large at death. The condition, therefore, during suprarenal insufficiency is that of a compensated alkali deficit which in the advanced stages becomes uncompensated (personal communication from Dr. K. Stuart Hetzel).

cortical hormone, recall particularly the studies of Gamble and his coworkers in the dog concerning loss of pancreatic juice due to drainage from a Pawlow fistula (16, 17). The similarity in the symptoms of the animals which they studied to those observed during the course of insufficiency in the suprarenalectomized dog is evident. The greater rapidity of the appearance of serious symptoms on a meat diet without added salt, in which the fixed base is in large part potassium, and hence unsuited to the repair of plasma and interstitial fluid loss was striking and also in keeping with the effects in the suprarenalectomized dogs deprived of extract.

Since the loss of sodium and of chlorides must come chiefly from the body fluids in which they principally occur, namely the blood plasma and interstitial tissue fluid, it follows that the water loss from the parenchymatous tissues, in which potassium is the principal base, should be relatively slight. Such appears to be the case in the animal dying in insufficiency. There is no appreciable reduction in the water content of liver or of muscle tissue in the suprarenalectomized dog or rat dying of insufficiency, despite the marked loss of weight. Analyses of such tissue which we have made from three suprarenalectomized dogs and nine suprarenalectomized rats, killed during severe insufficiency following the cessation of extract, when compared with like tissues obtained from a control series composed of equal number of normal animals, shows an average difference in water content of less than 2 per cent.

The increased excretion of urinary nitrogen and phosphorus, which follows resumption of injections of cortical extract, indicates an actual destruction of protoplasm and a reduction in its total mass during insufficiency. Destruction of muscle tissue is suggested by the changes in creatine and creatinine excretion as well.

The question now arises as to whether the dehydration, used in its broader sense, to embrace not only loss of water, but loss of electrolytes from the interstitial body fluids and blood plasma, is a sufficient explanation for the failure of the several physiological processes which we have described in our earlier papers (1, 2), and the ultimate cause of death in suprarenal insufficiency. We cannot at the present time accept this explanation as proven, but we believe the evidence presented indicates its importance and renders superfluous the various theories of intoxication which have been so frequently put forward. Studies of the effect of sodium chloride in the acute insufficiency of Addison's disease support this view (18).

The experience of numerous authors indicates clearly that all attempts to support the life of suprarenalectomized animals by sodium chloride alone have been unsuccessful. The cortical hormone, at least in minimal amounts, is indispensable for this purpose. It is possible that in the particular species which we have studied, the dog, the changes due to dehydration occur early, and mask other equally important changes due to lack of the cortical hormone, which make their appearance more slowly in this animal.

SUMMARY

A characteristic alteration in the electrolyte structure of the blood plasma of the suprarenalectomized dog occurs when injections of cortical extract are stopped. This alteration progresses during the course of the suprarenal insufficiency, parallel with the hemoconcentration and the loss in weight. When injections of cortical extract are resumed, the electrolyte structure returns to its original form, the alterations paralleling the dilution of the blood and the return of the body weight to its original level.

The hemoconcentration, with the resulting physiological changes which take place in the suprarenalectomized dog after the cessation of cortical extract injections, is associated with a loss of sodium and chloride, accompanied by their proper complement of body water, by way of the kidney. Since this effect is produced in the suprarenalectomized animal, well nourished and in excellent condition, solely by cessation of injections of the cortical hormone, and since the reverse process of repair of the electrolyte and water losses can be effected solely by resumption of extract injections, it follows that all of the observed phenomena are due to this cause, and to this alone. It can be concluded that one function of the cortical extract in the suprarenalectomized dog is that of participation in the regulation of the sodium and chloride metabolism, and consequently, of the balance and distribution of water. The loss of water, in the absence of the cortical hormone, is sustained partly by the blood plasma, but to a far greater extent by the interstitial body fluid. The available evidence points to the kidney as the locus of this regulatory function of the cortical hormone.

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EXPERIMENTAL

In the work described at this time four lines of investigation were followed. In the first place, an effort was made to determine what pathological changes are induced by vaccine virus in the brains of immune, partially immune, and normal monkeys. Then it seemed advisable to ascertain whether a testicular extract, Reynals' factor (11, 12), is capable of inducing vaccine virus to produce an acute disseminated encephalomyelitis in monkeys. Furthermore, inasmuch as it has been stated (13, 14) that repeated injections of normal brain tissues cause paralyses in rabbits, an attempt was made to determine whether inoculations of such tissues in monkeys would cause an acute disseminated encephalomyelitis. Finally, it seemed of interest to find out whether vaccine virus would cause an acute disseminated encephalomyelitis in monkeys that had received repeated injections of emulsions and extracts of normal rabbit brains.

Methods and Materials

Monkeys.—All monkeys (*Macacus rhesus*) used in the experiments were healthy and approximately half-grown.

Virus.—The dermal strain of vaccine virus employed was originally obtained from the New York City Board of Health (15). It has been freed from bacteria and has been propagated in rabbits and by means of cultivation *in vitro* in a mixture of viable chick embryo tissue and Tyrode's solution (15). It follows that none of the results reported in this paper can be ascribed to the action of bacterial contaminants. When intracranial injections of vaccine virus were made, the monkeys in the majority of instances received known amounts of the same testicular virus emulsion, No. 3285, preserved in 50 per cent glycerol, the titer of which in rabbits was 1–1,000,000. Culture virus was usually employed for dermal inoculations of the monkeys. The reason for the use of virus propagated in chick embryo tissue will be apparent when the experiments are described in detail.

Fresh Brain Emulsions.—The fresh brain emulsions injected into monkeys were prepared in the following manner: One normal rabbit brain was thoroughly ground with alundum in a mortar. 40 cc. of Locke's solution and 10 cc. of 95 per cent alcohol were added. Then the emulsion was centrifuged at speed 5 for 3 minutes. 3–5 cc. of the supernatant material were injected into each monkey. Fresh emulsions were made for each set of inoculations. The sterility of the materials employed was tested by cultures.

Brain Extracts.—The brains of 4 rabbits that had been exsanguinated were removed and thoroughly ground in a mortar, without an abrasive. This material was then placed in a large flask and extracted for 4 days at 37°C. with 300 cc. of

95 per cent alcohol. The alcohol was drawn off and saved. Then 300 cc. of ether were added to the brain tissue and extraction was allowed to take place at 37°C. for 6 days. The ether was removed and allowed to evaporate under the influence of heat and vacuum until only 20 cc. of a "soapy-looking" material remained. To this material were added the 300 cc. of alcohol with which the first extraction was made. The "soapy-looking" material went into solution. By means of heat (70°C.) and vacuum the volume of the mixed extracts was reduced to 150 cc. The concentrated alcohol-ether extract was stored in a cold room kept at 0°C. At this temperature a white waxy sediment appeared in the extract; but when the temperature of the extract was raised to 70 or 80°C., the sediment again went into solution. For the injection of each monkey 1 cc. of the alcohol-ether extract heated to 70–80°C. was added to 3 or 4 cc. of sterile distilled water. The resulting mixture consisted of a milky-looking fluid with the appearance of a Wassermann antigen. All of the prepared materials were tested for the presence of bacteria by means of cultures.

Injections of Brain Emulsions and Extracts.—The brain emulsions and extracts were repeatedly injected intramuscularly in monkeys, the animals receiving 3 inoculations a week consisting either of 2 emulsions and 1 extract or of 2 extracts and 1 emulsion.

Testicular Extract (Reynals' Factor).—The Reynals' factor used in the experiments consisted of a bacteria-free Berkefeld N filtrate of a distilled water (15 cc.) extract of desiccated testicular tissue (7.5 gm.).

Autopsies.—Complete autopsies were performed on all monkeys that died or that were sacrificed. Cultures to test the sterility of all brains were made.

Stains.—Sections from different parts of the cerebrum, cerebellum, pons, and cord were stained with hematoxylin and eosin, according to Marchi's method, and according to Kulschitzky-Wolter's modification of Weigert's myelin sheath stain.

Effect of Vaccine Virus on the Normal, the Partially Immune, and the Immune Brain of the Monkey

It has been stated (9) that vaccine virus introduced into the cisterna magna of a normal monkey produces a disease somewhat similar to postvaccinal encephalomyelitis in man. Furthermore, it has been suggested (16) that the acute disseminated encephalomyelitis following certain virus maladies including vaccinia is an hyperergic phenomenon (16). In regard to vaccinia, such an hypothesis is easily tested by experimentation in animals. Consequently, we decided to see what clinical and pathological manifestations are induced by the introduction of vaccine virus into the cisterns of monkeys during the process of immunization against the active agent. Inasmuch as normal monkeys were used as controls, an opportunity was also afforded to study the effect of the virus on the brains of non-immune animals.

The 9 experiments performed to determine the effect that vaccine virus has on the brains of normal, partially immune, and immune monkeys have been divided into 2 groups. The animals of the first group received intracranially 1 cc. of a 1–20 dilution of a rabbit testicular virus, while those of the second group received 1 cc. of a 1–5 dilution of the same virus.

Group I.—Group I consisted of 5 experiments in which 13 monkeys were used, 5 of which were controls. 8 monkeys were inoculated on the skin with vaccine virus that had been cultivated in a mixture of chick embryo tissue and Tyrode's solution. The virus was smeared on 2 large areas of scarified skin. On the 2nd, 4th, 6th, and 10th days after the dermal inoculations either 1 or 2 of the monkeys together with a control received intracisternally 1 cc. each of 1–20 dilution of a rabbit testicular vaccine virus, No. 3285. In addition to these animals, 1 monkey dermally vaccinated 6 days previously and 1 normal monkey received 1 cc. each of the testicular virus in the right parietal lobe. All of the control animals died of a meningitis. The control monkey that received the virus in the right parietal lobe also had a partial paralysis of the left arm and leg. The 2 monkeys that had been vaccinated 2 days prior to the cisternal inoculations died of a meningitis similar to that of the controls. The 2 monkeys that had been vaccinated 4 days prior to the cisternal inoculations had a mild meningitis from which they promptly recovered. The animals vaccinated 6 and 10 days prior to the cisternal inoculations showed no clinical evidences of cerebral involvement. None of the test and control animals showed any manifestations suggestive of an acute disseminated encephalomyelitis. The results of these experiments are summarized in Table I.

Group II.—The monkeys in Group I vaccinated on the skin developed a refractory state to the cisternal inoculations of a 1–20 dilution of the testicular virus so rapidly that we decided to do another series of experiments in which 1–5 dilutions of the same virus emulsion were employed. This set of 4 experiments represents Group II in which 8 monkeys were used. 4 monkeys were vaccinated on the skin with culture virus similar to that used for the animals in Group I. On the 5th, 7th, 10th, and 12th days after vaccination, 1 vaccinated and 1 control monkey received intracisternally 1 cc. each of a 1–5 dilution of testicular virus, No. 3285. All of the control animals died of a meningitis. The monkeys that had been vaccinated 5 and 7 days prior to the cisternal inoculations also died of a meningitis similar to that of the controls. The monkey that had been vaccinated 10 days prior to the cisternal inoculation developed a mild meningitis from which it promptly recovered, while the one vaccinated 12 days prior showed no evidences of cerebral involvement. None of the animals developed signs suggestive of an acute disseminated encephalomyelitis. The results of the experiments are summarized in Table II.

Pathology.—The pathological findings in the brains of the monkeys that died following the cisternal inoculations of vaccine virus consisted of a non-purulent meningitis most intense around the pons, cerebellum, and cord. A slight amount of perivascular infiltration and glial reaction was seen in the superficial portions of the brain. The response of the partially immune brains to the infection differed in no obvious way from that of the normal brains. In no instance was an acute disseminated encephalomyelitis with perivascular demyelination encountered. Figs. 1–4 portray the pathology of vaccinal meningitis and encephalitis in monkeys.

TABLE I

Summary of Experiments in Which Each Monkey Received Intracranially 1 Cc. of a 1–20 Dilution of a Rabbit Testicular Vaccine Virus, No. 3285

Experiment No.	Monkey No.	No. of days between dermal vaccination and intracranial inoculation	Site of intracranial inoculation	Result	Remarks
I	1	2	Cisterna magna	Died, 5 days	Signs of meningitis
	2	2	“ “	“ 6 “	“ “ “
	3	No dermal vaccination	“ “	“ 5 “	“ “ “
II	4	4	“ “	Survived	Signs of a mild meningitis
	5	4	“ “	“	“ “
	6	No dermal vaccination	“ “	Died, 6 days	Signs of meningitis
III	7	6	Right parietal lobe	Survived	No signs of cerebral involvement
	8	No dermal vaccination	“ “	Died, 6 days	Signs of meningitis, weakness in left leg and arm
IV	9	6	Cisterna magna	Survived	No signs of cerebral involvement
	10	No dermal vaccination	“ “	Died, 8 days	Signs of meningitis
V	11	10	“ “	Survived	No signs of cerebral involvement
	12	10	“ “	“	“ “
	13	No dermal vaccination	“ “	Died, 6 days	Signs of meningitis

The results of the experiments described above and summarized in Tables I and II indicate that the brain of a vaccinated monkey rapidly becomes refractory to the virus, probably just as rapidly as does the skin. Furthermore, it seems evident that vaccine virus injected intracisternally in monkeys produces a meningitis accompanied by a mild superficial encephalitis, and not an acute disseminated encephalomyelitis with perivascular demyelination.

TABLE II

Summary of Experiments in Which Each Monkey Received Intracranially 1 Cc. of a 1–5 Dilution of a Rabbit Testicular Vaccine Virus, No. 3285

Experiment No.	Monkey No.	No. of days between dermal vaccination and intracranial inoculation	Site of intracranial inoculation	Result	Remarks
VI	14	5	Cisterna magna	Died, 3 days	Signs of meningitis
	15	No dermal vaccination	" "	" 5 "	" " "
VII	16	7	" "	" 4 "	" " "
	17	No dermal vaccination	" "	" 4 "	" " "
VIII	18	10	" "	Survived	Signs of a mild meningitis
	19	No dermal vaccination	" "	Died, 3 days	Signs of meningitis
IX	20	12	" "	Survived	No signs of cerebral involvement
	21	No dermal vaccination	" "	Died, 5 days	Signs of meningitis

Effect of Testicular Extracts (Reynals' Factor) on Vaccinal Infections in the Brains of Monkeys

It has been shown (11, 12) that testicular extracts in some manner enhance the action of vaccine virus. In view of this fact, we decided to determine whether by means of such an extract vaccine virus could be induced to cause an acute disseminated encephalomyelitis in monkeys.

Experiment X.—In this experiment we attempted to find out (1) whether a testicular extract injected into the cistern of a monkey would cause vaccine virus placed on the skin of the thorax to invade the brain and (2) whether a mixture of testicular extract and vaccine virus injected into the cistern of a monkey vaccinated on the skin 3 days previously would produce a marked encephalitis as well as a meningitis.

Monkey 22 received intracisternally 1 cc. of Reynals' factor and showed no signs of cerebral involvement. Monkey 23 was vaccinated on the skin with culture virus. 2 and 7 days after the dermal vaccination the animal received intracisternally 1 cc. of Reynals' factor and showed no signs of cerebral involvement. Monkey 24 was vaccinated on the skin with culture virus and 3 days later received intracisternally a mixture of a 0.5 cc. of Reynals' factor and a 0.5 cc. of a 1–20 dilution of testicular virus, No. 3285. The animal developed a stiff neck, ataxia, and spasticity, and was sacrificed 7 days after the cisternal inoculation. Sections of the brain and cord revealed pictures similar to those described earlier in the paper. Monkey 25 received intracisternally a mixture of a 0.5 cc. of Reynals' factor and a 0.5 cc. of a 1–20 dilution of testicular virus, No. 3285. The animal developed a stiff neck, ataxia, and weakness of the arms, and was sacrificed when found moribund on the 6th day after inoculation. Sections of the brain and cord revealed pictures similar to those described earlier in the paper.

The evidence obtained from Experiment X indicated that we would be unable by means of Reynals' factor and vaccine virus to produce an acute disseminated encephalomyelitis in monkeys. Consequently we pursued the matter no further.

Effect in Monkeys of Repeated Injections of Emulsions and Extracts of Normal Rabbit Brains

During antirabic vaccination an occasional patient develops signs and symptoms of involvement of the central nervous system (17, 18). Some of these patients die and at autopsy a myelitis or an encephalitis is found. In many instances, however, the meager descriptions of the pathological changes in the brain and cord leave one in doubt as to their nature. Nevertheless, sufficiently accurate and complete descriptions (18–20) have been made to indicate that in certain instances the lesions are similar to those seen in postvaccinal encephalomyelitis. The cause of this rare complication of antirabic vaccination is unknown. Certain workers have suggested, however, that it is produced by the repeated injections of brain or cord tissues in the antirabic vaccine (13, 18). Indeed, several workers (13) believe that they have produced the disease experimentally in rabbits by means of repeated injections of normal brain tissues. Nevertheless, Hurst (14) recently found that no obvious pathological changes were present in the brains and cords of rabbits that became paralyzed while receiving repeated injections of normal brain tissues. Inasmuch as monkeys had not been used for this type of work, we decided to find out what effect repeated injections of emulsions and extracts of normal rabbit brains would have on such animals.

Experiment XI.—8 healthy half-grown monkeys (*Macacus rhesus*) received repeated inoculations (3 a week) of emulsions and extracts of normal rabbit brains. The emulsions and extracts were prepared and administered to the animals in the manner described under the heading of Methods and Materials.

Each of 2 monkeys, Nos. 26 and 27, received 14 injections, 5 of emulsions and 9 of extracts. Both monkeys remained well and were used for Experiment XII in which the brains and cords were examined and found to show no perivascular demyelination.

Each of 3 monkeys, Nos. 29, 32, and 33, received 93 injections, 52 of emulsions and 41 of extracts. The 3 animals remained healthy and were used for Experiment XIII in which the brains and cords were examined and found to show no perivascular demyelination.

Monkey 30 received, between May 26, 1931, and Dec. 14, 1931, 84 injections, 46 of emulsions and 38 of extracts. On Nov. 9, it was noticed that the monkey kept its head turned to the left, and that there was a tendency for the chin to rest on the left shoulder. The animal at this time began to lose weight and grow weak. On Dec. 2, the monkey seemed ataxic, climbed about the cage with difficulty, and became tired quickly. The animal gradually grew worse and was sacrificed on Dec. 18, 1931. At no time were there any signs suggestive of tuberculosis. At autopsy all the organs macroscopically appeared normal. No evidence of tuberculosis was seen. Cultures of the brain remained sterile. Sections stained with hematoxylin and eosin revealed a slight amount of perivascular infiltration in the cerebrum and cord, a marked perivascular infiltration in the midbrain, pons (Figs. 5 and 7), medulla, and cerebellum (Fig. 6). In certain instances, the cells seemed to be collected in the perivascular spaces, while in others there was definite glial reaction extending out from the vessels into the substance of the brain. Some areas not definitely associated with vessels (Fig. 5) showed a similar glial reaction. In places the lesions had a granulomatous appearance, and an occasional giant cell (Fig. 9) was found. In spite of the fact that mononuclear cells predominated in the cellular reaction a surprisingly large number of eosinophilic polymorphonuclear elements were also found. In certain areas the meninges (Fig. 9) of the cerebellum were involved and showed an infiltration of mononuclear cells. Marchi and Weigert stains revealed definite changes in or loss of myelin sheaths in the neighborhood of vessels in the pons (Fig. 10) and cerebellum (Fig. 8). No degeneration of spinal cord tracts was seen.

Monkey 31 received, between May 26, 1931, and Oct. 10, 1931, 52 injections, 28 of emulsions and 24 of extracts. On July 20, an abscess formed at the site of one of the inoculations. From that time until Aug. 10 the injections were discontinued. After the abscess healed, the inoculations were again made at the rate of 3 a week. On Oct. 9, the animal appeared weak and dragged its left leg. Oct. 14, the animal was weaker, left arm and leg were partially paralyzed leading to incoordination of movement particularly noticeable when the monkey made attempts to climb up the side of the cage. Oct. 16, the animal was sacrificed and autopsied immediately. All the organs seemed normal except the brain. No evidence of tuberculosis was found. The brain was congested and on section the white substance of the right parietal lobe had a moth-eaten appearance. A Sudan III preparation from this area showed a large number of fat-containing cells around the vessels. Cultures made from the brain remained sterile. Sections of the brain stained with hematoxylin and eosin revealed a marked infiltration of cells in the white substance of the right parietal lobe (Figs. 12–15) and a slight infiltration in the left parietal lobe. In general, the cells seemed to be collected around the vessels (Figs. 12, 15) but they also extended well out into the substance of the brain (Fig. 13, 14). At times the cells assumed a palisade arrangement around minute areas of necrosis in such a manner as to be suggestive of granulomatous lesions. Mononuclear cells, particularly glial elements, pre-

TABLE III

Summary of the Results of Experiment XI in Which Monkeys Received Repeated Injections of Emulsion and Extracts of Normal Rabbit Brains

Monkey No.	No. of injections of brain emulsions	No. of injections of brain extracts	Results
26	5	9	Negative. Used in Experiment XII
27	5	9	" " " " XII
28	24	26	Died suddenly after 50th injection. Brain and cord negative
29	52	41	Negative. Used in Experiment XIII
30	46	38	Developed a tendency to hold chin on left shoulder, ataxia, and weakness. Sacrificed. Section showed involvement of midbrain, pons, cerebellum, and medulla, with perivascular demyelination
31	28	24	Developed ataxia, general weakness, and paresis of left leg and arm. Sacrificed. Section through right parietal and temporal lobes showed marked involvement of white matter with perivascular demyelination. Tract degeneration in cord
32	52	41	Negative. Used in Experiment XIII
33	52	41	" " " " XIII

dominated in the reaction. Nevertheless, a surprisingly large number of eosinophilic polymorphonuclear elements were found. Marchi and Weigert preparations showed marked changes in or loss of the myelin sheaths in the right parietal lobe (Fig. 11). Slight pathological changes of a similar nature were also found in the left parietal lobe. Marchi preparations of the cord revealed degeneration of myelin in both of the lateral pyramidal tracts and both of the anterior median columns.

From the results of Experiment XI (Table III) it appears that of 8 healthy half-grown monkeys (*Macacus rhesus*) receiving repeated injections of emulsions and alcohol-ether extracts of normal rabbit brains, 5 developed no clinical signs of involvement of the central nervous system and at autopsy showed no evidences of perivascular demyelination, 1 suddenly died after an injection and had no demonstrable pathological changes in the central nervous system, and 2 developed clinical signs of involvement of the central nervous system in which sections revealed an inflammatory reaction accompanied by demyelination. The significance of the findings in these 2 monkeys will be discussed in another part of the paper.

Effect of Vaccine Virus on Monkeys Receiving Repeated Injections of Emulsions and Extracts of Normal Rabbit Brains

Having studied the effect of vaccine virus on the brains of normal, partially immune, and immune monkeys, and having noted the effect in monkeys of repeated injections of emulsions and alcohol-ether extracts of normal rabbit brains, we decided to investigate the reaction of monkeys to the combined action of vaccine virus and emulsions and extracts of normal brain tissue. This matter was dealt with in Experiments XII and XIII.

Experiment XII.—Monkey 26, between May 26, 1931, and July 1, 1931, received 14 injections, 5 of emulsions of fresh rabbit brain and 9 of alcohol-ether extracts of brain tissue. Shortly after the last injection the monkey was inoculated in 2 places on the skin with vaccine virus propagated in tissue cultures. 2 days after the dermal vaccination, a cisternal puncture was made, fluid was removed, and 0.5 cc. of a 1–20 Locke's solution dilution of the alcohol-ether extract of brain tissue plus 0.5 cc. of a 1–20 Locke's solution dilution of culture vaccine virus was injected. Typical lesions developed at the sites of dermal vaccination. As a result of the cisternal injection of virus, the animal developed signs of meningitis and was sacrificed 10 days after the inoculation. Examination of the brain and cord showed a meningitis similar to that described earlier in the paper. No evidence of perivascular demyelination was found in Marchi and Weigert preparations. Monkey 27, control for No. 26, received, between May 26, 1931, and July 1, 1931, 14 injections, 5 of brain emulsions and 9 of brain extracts. This animal was not vaccinated on the skin, but received an inoculation in the cisterna magna similar to that given Monkey 26. The animal developed meningitis and was sacrificed 9 days after the cisternal inoculation. Examination of the brain and cord showed a meningitis similar to that found in Monkey 26.

Experiment XIII.—Each of 3 monkeys, Nos. 29, 32, and 33, received, between May 26, 1931, and Jan. 11, 1932, 93 injections, 52 of brain emulsions and 41 of brain extracts. A few days after the last injection the animals were inoculated in the following manner: Monkey 29, vaccinated on the skin with rabbit testicular vaccine virus, received intravenously 1 cc. of a 1–5 dilution of culture vaccine virus and intracerebrally 0.25 cc. of a 1 per cent emulsion of fresh rabbit brain. Monkey 32, vaccinated on the skin with rabbit testicular vaccine virus, received intravenously 1 cc. of a 1–5 dilution of culture vaccine virus plus 1 cc. of a 1 per cent emulsion of fresh rabbit brain mixed with 3 cc. of Locke's solution; a cisternal puncture was made and 2 cc. of fluid were withdrawn. Monkey 33, vaccinated on the skin with rabbit testicular vaccine virus, received intravenously 1 cc. of a 1–5 culture vaccine virus; a cisternal puncture was made, 1.5 cc. of fluid were withdrawn, and 0.35 cc. of a 1 per cent emulsion of fresh rabbit brain were injected. The 3 monkeys developed typical vaccinal lesions at the sites of skin inoculation, but never showed signs of central nervous system involvement. Monkeys 29, 32, and 33 were sacrificed 18, 17, and 16 days respectively after inoculation. Examination of the brains and cords revealed no evidences of meningitis, encephalitis, or perivascular demyelination.

From Experiments XII and XIII no evidence was obtained to indicate that the combined action of vaccine virus and an emulsion of fresh rabbit brain is capable of producing perivascular demyelination in monkeys that had received repeated injections of emulsions and alcohol-ether extracts of normal rabbit brains.

DISCUSSION

The acute disseminated encephalomyelitis that occasionally follows Jennerian prophylaxis, smallpox, measles, and antirabic vaccination is now a well recognized pathological picture (1, 2, 3, 20) and is particularly characterized by perivascular demyelination. Indeed, the perivascular demyelination is such an important part of the process that all investigators attempting to produce the disease experimentally should study their material by means of Marchi and Weigert stains as well as by the ordinary stains usually employed. In the past certain workers have failed to do so, and this fact leaves one in doubt concerning the results actually obtained by them.

The results of the work reported in the first part of the paper clearly show that vaccine virus, free from bacteria, injected into the cisterna magna or into the parietal lobe of a monkey leads to a fatal disease. These findings are in accord with those of Eckstein (9) and Hurst and Fairbrother (4). The pathological picture is that of a severe meningitis accompanied by a mild encephalitis involving the superficial portions of the brain. No evidence was obtained to indicate that the direct action of vaccine virus on the brain of a normal monkey is capable of producing an acute disseminated encephalomyelitis with perivascular demyelination. In this respect our results are different from those reported by Clearkin (6) and McIntosh and Scarff (5).

The results of our work dealing with the effect of vaccine virus on the brains of partially immune and immune monkeys lend no support to the idea that postvaccinal encephalomyelitis is an hyperergic phenomenon (16). We did find, however, that the brain of a monkey vaccinated on the skin rapidly becomes refractory to the active agent placed in the cisterna magna or in the parietal lobe. Indeed, the refractory state is evident by the 4th day and quite marked by the 6th day after dermal vaccination (Table I).

A testicular extract prepared in the manner described by Reynals enhances the activity of vaccine virus. It has been suggested that the enhancement is due in part to an increased permeability of susceptible cells (12). In Experiment X, the Reynals' factor with and without vaccine virus was placed in the cisterns of monkeys, some of which had, while others had not been vaccinated on the skin. This was done in the hope that the testicular extract would break down the barrier between the subarachnoid space and brain or between the blood stream and brain in such a way as to permit the vaccine virus to produce an acute disseminated encephalomyelitis. Our hopes were not realized, however, because such an encephalomyelitis failed to be induced in this manner.

The results of repeated injections of brain emulsions and brain extracts into monkeys are interesting. The significance of the findings, however, is not clear. One animal died suddenly after an inoculation, but showed no lesions in the brain and cord to account for the death. This is in accord with observations (14) that sudden deaths without obvious cause occur in rabbits receiving repeated inoculations of brain emulsions. Two monkeys developed clinical signs of involvement of the central nervous system. Examination of one of the animals revealed an inflammatory reaction accompanied by perivascular demyelination in the midbrain, pons, medulla, and cerebellum. Examination of the other animal brought to light pathological changes in the cerebrum suggestive of, but different from, those seen in Schilder's disease. Inasmuch as two of eight monkeys receiving repeated injections of brain emulsions and brain extracts developed central nervous system lesions with destruction of myelin sheaths, one might be tempted to ascribe the pathological changes to the treatment received by the animals. Nevertheless, the lesions were not typical of disseminated encephalomyelitis. Furthermore, from reports in the literature (21–26) it appears that monkeys may suffer from spontaneous demyelinating diseases of the central nervous system. The picture in our monkeys, however, is not identical with any of those already recorded. Consequently, at the moment we hesitate to state definitely what relation the treatment of our monkeys had to the disease found in them. The experiment should be repeated with a larger number of monkeys, and we intend to do so if the opportunity arises.

SUMMARY

No evidence was found to support the idea that vaccine virus placed in the cisterna magna is capable of producing an acute disseminated encephalomyelitis with perivascular demyelination either in normal or in partially immune monkeys.

A testicular extract (Reynals' factor) did not induce vaccine virus to cause an acute disseminated encephalomyelitis in monkeys.

Repeated intramuscular injections of brain extracts and brain emulsions into eight monkeys were followed in two instances by an inflammatory reaction, accompanied by demyelination, in the central nervous system. The exact relation of the injections to the disease of the nervous system is not clear.

The combined action of vaccine virus and an emulsion of fresh rabbit brain did not lead to the production of an acute disseminated encephalo-

myelitis in monkeys that had received repeated intramuscular injections of emulsions and alcohol-ether extracts of normal rabbit brains.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Vaccinal meningitis in the neighborhood of the pons and cerebellum. The exudate consists of fibrin and polymorphonuclear and mononuclear cells. H. and E. × 63.

Fig. 2. Inflammation of the meninges of the cord caused by vaccine virus. H. and E. × 63.

Fig. 3. Inflammation of the cerebral meninges induced by vaccine virus. The exudate immediately in contact with the surface of the brain consists of mononuclear cells and is suggestive of the type of reaction caused by the activity of vaccine

virus in the skin. The substance of the brain near the surface also enters into the reaction and displays a certain amount of perivascular infiltration. H. and E. × 250.

FIG. 4. Perivascular infiltration in the pons caused by vaccine virus placed in the cisterna magna. The perivascular exudate was rarely as marked as portrayed. H. and E. × 250.

PLATE 2

FIG. 5. Lesions in the pons of Monkey 30. H. and E. × 63.

FIG. 6. Lesions in the cerebellum of Monkey 30. H. and E. × 63.

FIG. 7. Perivascular infiltration in the pons of Monkey 30. H. and E. × 250.

FIG. 8. Demyelination in cerebellum of Monkey 30. Modified Weigert stain. × 37.

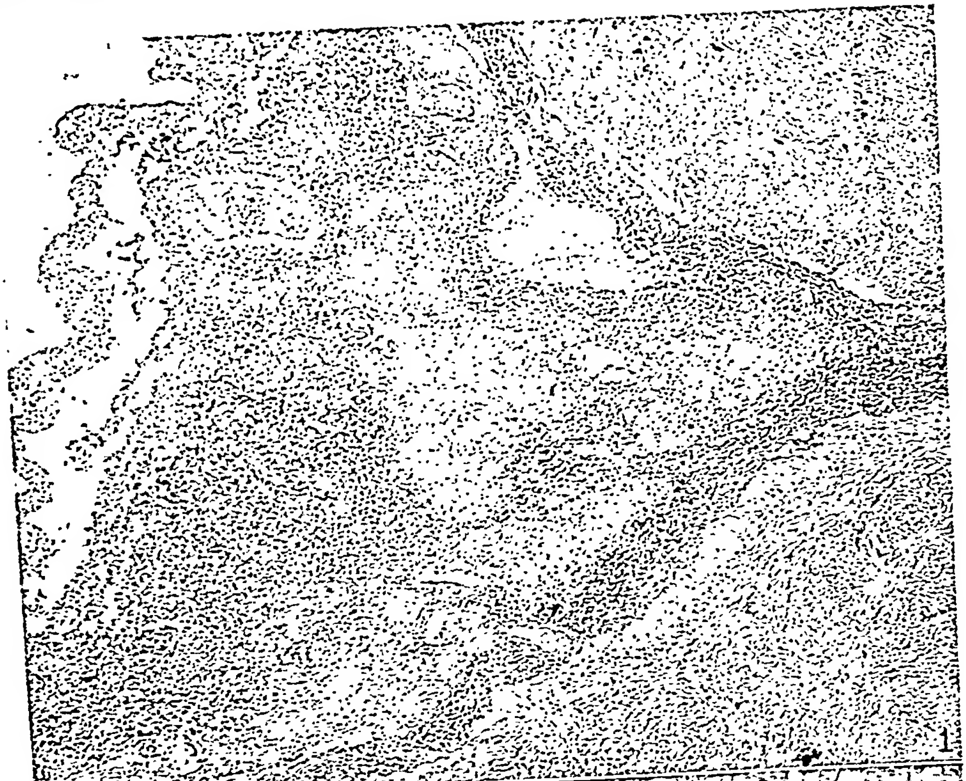
FIG. 9. Giant cells in cerebellum, and thickened cerebellar meninges infiltrated with mononuclear cells. Monkey 30. H. and E. × 250.

FIG. 10. Demyelination in pons of Monkey 30. Modified Weigert stain. × 37.

PLATE 3

FIG. 11. Demyelination in the right parietal lobe of Monkey 31. The picture is suggestive of Schilder's disease. Modified Weigert stain. × 37.

FIGS. 12–15. Lesions in the right parietal lobe of Monkey 31. Note the perivascular distribution. H. and E. × 250 and × 63.



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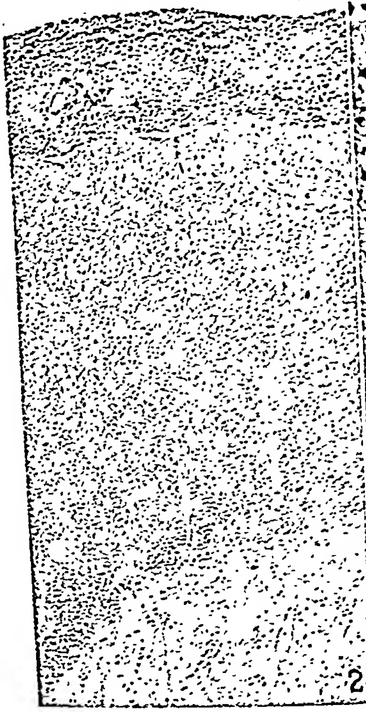

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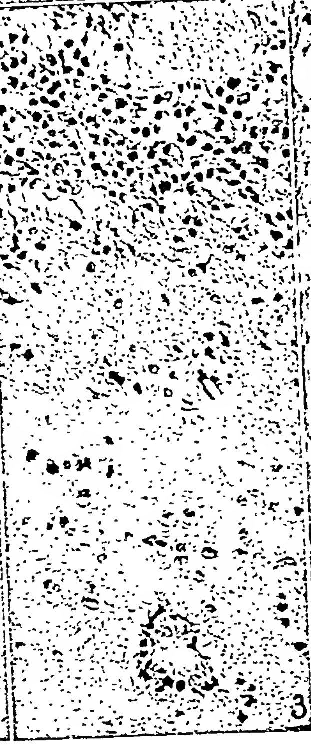

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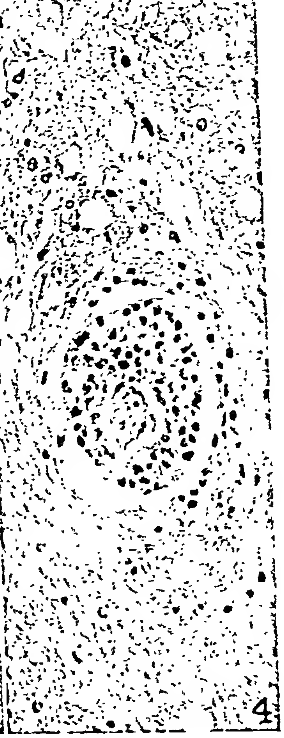

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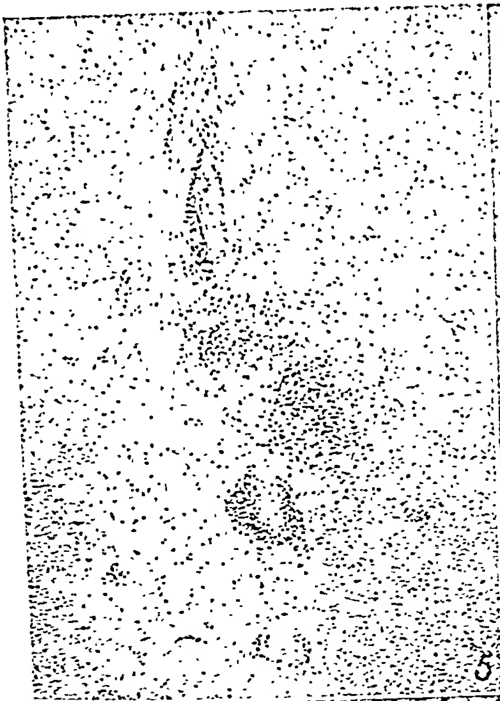
Photographed by Louis Schmidt

(Rivers *et al.*: Acute disseminated encephalomyelitis)



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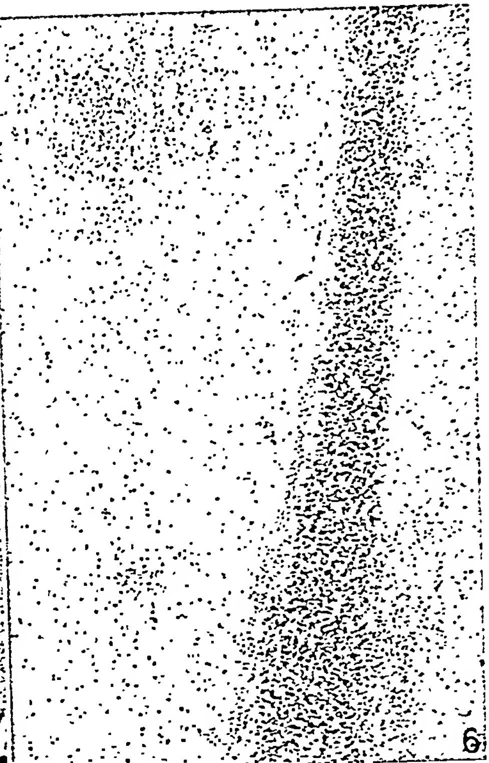

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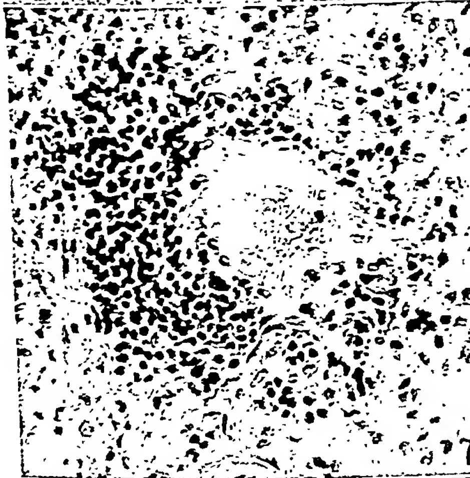

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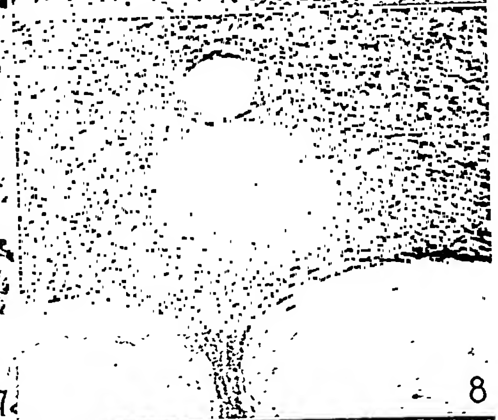

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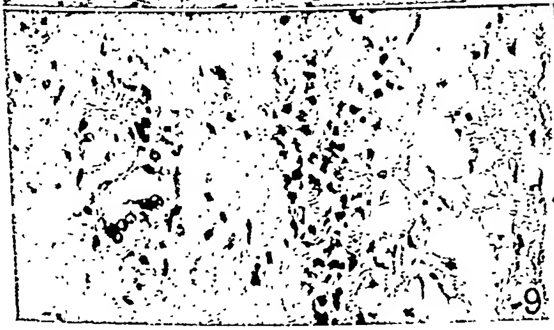

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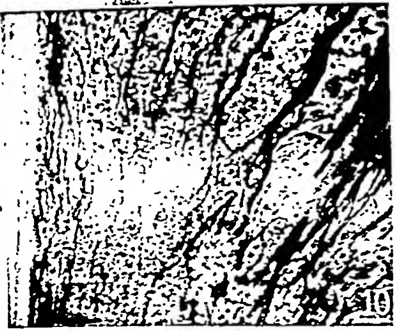

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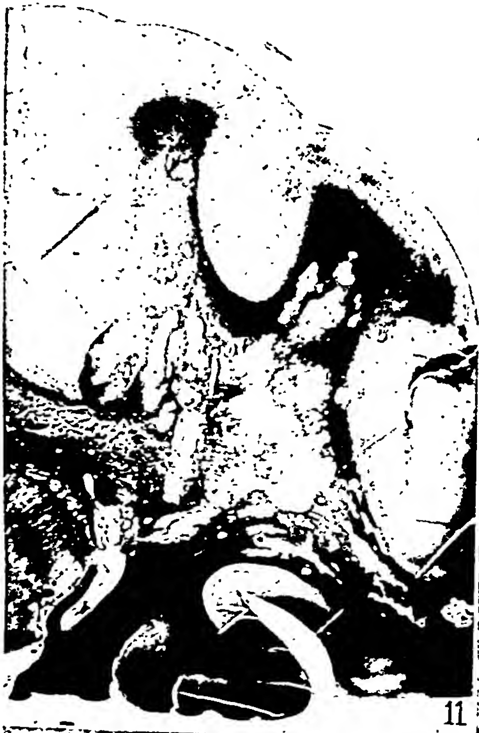
(Rivers *et al.*: Acute disseminated encephalomyelitis)



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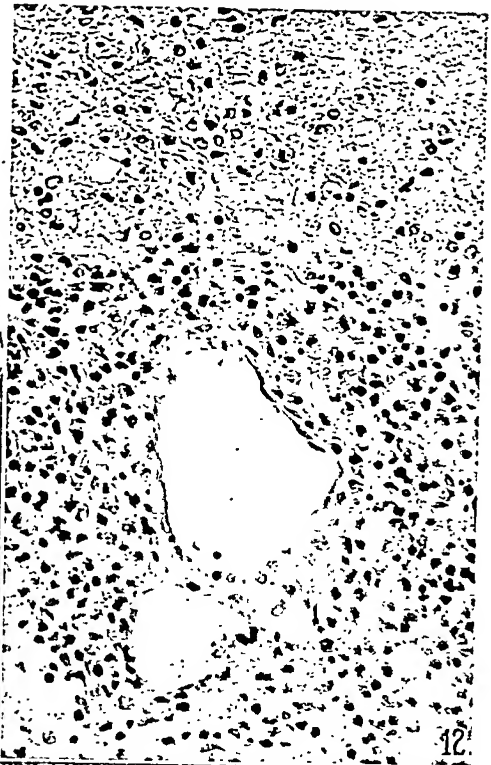

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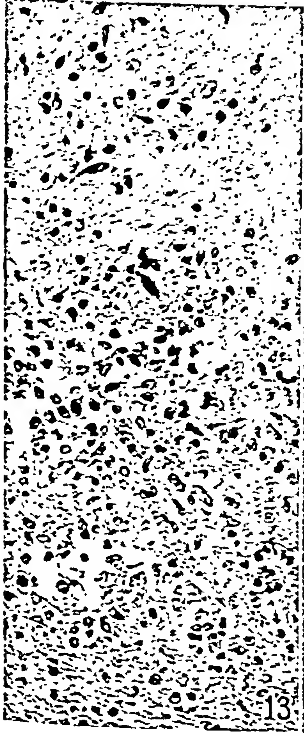

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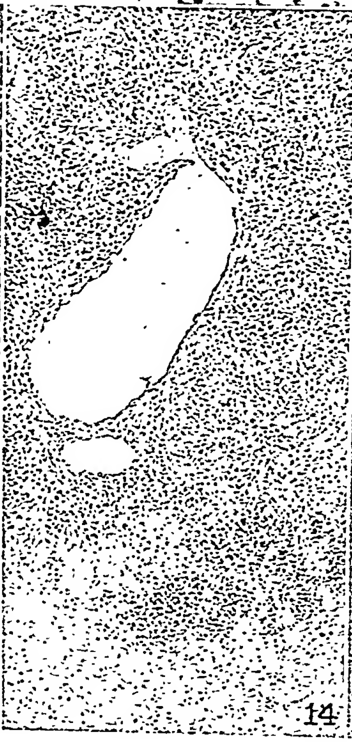

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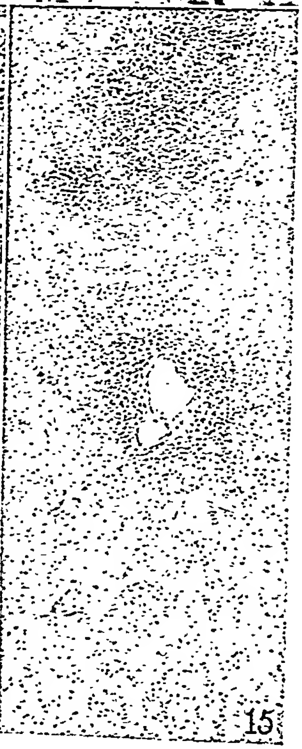

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THE ANTIGENIC RELATIONSHIP BETWEEN PROTEUS X-19 AND TYPHUS RICKETTSIAE

A Study of the Weil-Felix Reaction

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In a preceding paper from this laboratory, Zinsser and Castaneda (1) reported upon the development of a method by which reliable agglutination reactions could be obtained, with *Rickettsia* suspensions, in sera from convalescent typhus patients and in those of convalescent or vaccinated animals. The experiments carried out by this method showed a definite antigenic relationship between our own Mexican *Rickettsia* vaccines obtained from rats and the Weigl vaccines produced with European *Rickettsiae* obtained from infected lice. It was also found that there was parallelism between the agglutinating powers of the investigated sera for *Rickettsia* and for *Bacillus proteus* X-19. These results were in keeping with the immunologic studies on typhus fever published from this laboratory during recent years and with the well known specificity of the Weil-Felix reaction in the several types of *Rickettsia* infection. The method furnished an opportunity for a more precise investigation of the antigenic mechanism responsible for this specific relationship.

The experiments described below represent agglutinin absorption studies applied to this problem.

Technique

The materials used for agglutination were the following: (1) Weigl's phenolized vaccines, which consist of triturated suspensions of *Rickettsia prowazeki* obtained from the intestines of lice infected with European typhus. (2) Suspensions of Mexican *Rickettsiae* obtained from rats by the method described. (3) A non-motile "O" culture of *Bacillus proteus* X-19. The suspensions of this organism used were made from fresh agar slants in every case.

The agglutinating sera used were as follows: (1) European and Mexican convalescent typhus sera. (These were unfortunately quite old.) (2) A serum from an endemic case of typhus occurring in Boston. (3) The serum of a horse immunized with formalinized Mexican *Rickettsiae.* (4) The sera of rabbits immunized with the stock strain of *Bacillus proteus* X-19.

Absorptions were carried out by the addition of thick suspensions of either *Bacillus proteus* X-19 or of the Mexican *Rickettsiae* to sera diluted two to four times with salt solution. In the experiments presented in Table III, the sera were diluted to from 1:30 to 1:75 before absorption. The mixtures were kept in a water bath at 37°C. for from 3 to 4 hours and were then allowed to stand at room temperature overnight. Controls of similarly diluted sera—unabsorbed—were, in every case, identically treated. Before testing, the suspensions were centrifugalized at high speed in order to remove organisms.

TABLE I

Sera Absorbed with Proteus X-19

	Unabsorbed								Absorbed								Antigens
Serum dilutions..........	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:20	1:40	1:80	1:160	1:360	1:640	1:1,280	1:2,560	
Human typhus serum (Boston case)	4	4	4	4	3	1	—	—	2	1	—	—	—	—	—	—	*Proteus* X-19
	2	3	3	4	4	2			±	2	4	4	4	2			Weigl vaccine
		4	4	4	1	4	3			4	4	4	4	4	4	—	Mexican vaccine
Horse antityphus serum	4	4	4	1	—	—			—	—	—	—	—	—			*Proteus* X-19
				3	3	2			4	4	4	3	3	2			Weigl vaccine
Normal horse serum		2	—	—													*Proteus* X-19
		—	—														Weigl vaccine

4 = ++++, or complete. The other numerals are roughly in proportion.

In observing the reactions, the ordinary macroscopic test was used for the *Proteus* agglutinations, the tubes being placed in a water bath at 37° to 40°C. for 2 hours, and then left at room temperature overnight before final readings were made.

The *Rickettsia* agglutinations were carried out by methods described in the paper referred to (1).

Experiment I.—In this experiment, we used the serum of a woman who was admitted to the Beth Israel Hospital in Boston early in the winter of 1932–33, suffering from endemic typhus fever. The history and the clinical course were typical and the Weil-Felix reaction titrated up to 1:640. Agglutination of both the European and the Mexican *Rickettsiae* in this case ran almost parallel with the Weil-Felix reaction, as will be noted from Table I. The fact that this serum was not from an epidemic Mexican or European case somewhat weakens the value of our experiment, since we were not able to obtain a virus from the patient, probably because our attempt to do this was made too late in the disease.

In the same experiment, we similarly absorbed our antityphus horse serum, controlling this part of the experiment with normal horse serum.

Table I illustrates the results.

Proteus X-19 removed, from the human serum as well as from the antityphus horse serum, practically all agglutinins for *Proteus* X-19, but did not affect the agglutination of the *Rickettsiae*.

In an experiment similar to our own, Krukowski (2) found that when European convalescent typhus serum was absorbed with *Proteus* X-19, the homologous agglutinins were removed, whereas those for the *Rickettsiae* were left unabsorbed. Our experiment fully confirms and extends this observation.

TABLE II

Sera Absorbed with Mexican Rickettsiae (Formalinized)

	Unabsorbed						Absorbed						Antigen
Serum dilutions	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	
Anti-*Proteus* Rabbit 1	4	4	4	4	4	—	4	4	4	4	4	—	*Proteus* X-19
Anti-*Proteus* Rabbit 2			4	2	—	—			4	2	—		*Proteus* X-19

Experiment II.—In this experiment, the sera of two rabbits which had been immunized with *Proteus* X-19 were absorbed with Mexican *Rickettsiae*. Table II illustrates the results of this experiment.

It is plain from Table II that the absorption of an anti-*Proteus* rabbit serum with Mexican *Rickettsiae* has practically no effect upon the anti-*Proteus* agglutinins.

Experiment III.—In this experiment, four human typhus sera, one from the Boston case, two from convalescent Mexican typhus patients and one from a

Polish case, were absorbed by the method described with formalinized Mexican *Rickettsiae.* These sera agglutinated *Proteus* X-19, as well as the Mexican *Rickettsiae,* in all instances except in that of the Polish case. This serum agglutinated Mexican *Rickettsiae* up to 1:160, as reported in the paper of Zinsser and Castaneda alluded to above (1). We did not have enough of the serum to include the desirable low dilution controls in the present instance.

Table III shows the results of this experiment.

It is apparent from Table III that absorption with Mexican *Rickettsiae* removed, either completely or almost completely, agglutinins both for *Proteus* X-19 and for our Mexican *Rickettsia* suspensions.

TABLE III

Sera Absorbed with Mexican Rickettsiae (Formalinized)

	Unabsorbed									Absorbed									Antigens
Serum dilutions....	1:60	1:100	1:200	1:300	1:400	1:500	1:600	1:800	1:1,600	1:60	1:100	1:200	1:300	1:400	1:500	1:600	1:800	1:1,600	
Human typhus	4	4	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—		*Proteus* X-19
Serum 1	4	4	4	1	4	4	4	4	—	2	±	—	—	—	—	—	—		Mexican vaccine
Human typhus			4	4			2					—	—			—			*Proteus* X-19
Serum 2			4	2			1		1			—	—						Mexican vaccine
Human typhus			4	4			4		2			2	2			1		—	*Proteus* X-19
Serum 3			2	3		4			3			2	—		—			—	Mexican vaccine
Human typhus			4	3			2		1			—	—			—		—	*Proteus* X -19
Serum 4			±	—								±	—						Mexican vaccine

Serum 1, from Boston case on the 12th day of disease. Serum 2, Mexican typhus. Serum 3, Mexican typhus. Serum 4, European typhus from Poland.

In past experiments, it has invariably appeared that animals immunized with *Proteus* X-19 possessed no agglutinating power for *Rickettsia* suspensions. This has been the experience of others and repeatedly noted in this laboratory. We have confirmed this observation with the Weigl vaccine, as well as with our own *Rickettsia* suspensions—fresh, formalinized and carbolized. Nevertheless, from the experiments described above, it seems reasonable to suppose that Mexican *Rickettsiae* contain an antigenic factor common to both

Rickettsia and to *Proteus* X-19. But the failure of detection of *Rickettsia* agglutinins in anti-*Proteus* serum would seem to be opposed to such a conception. In discussing this matter with Dr. Grinnell, who has studied the differences in agglutinability between heated and unheated *Bacillus typhosus*, we were led to investigate similar phenomena with *Rickettsiae*. The unexpected results are presented in the following experiment.

Rickettsia suspensions were prepared by washing the peritoneal cavity of X-rayed rats in the same way in which this is done in the preparation of typhus vaccine. Emulsions were made in saline and in formalinized saline. Both suspensions were heated at 75°C. for 30 minutes. Unheated material was reserved for controls.

TABLE IV

Agglutination of Mexican Rickettsiae by Anti-Proteus Serum

	Non-formalinized *Rickettsiae*										Formalinized *Rickettsiae*													
	Unheated					Heated					Unheated							Heated						
Serum dilutions.........	1:20	1:40	1:80	1:160	1:320	1:20	1:40	1:80	1:160	1:320	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Anti-*Proteus* Rabbit 1..............	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	2	3	3	3	3	1
Anti-*Proteus* Rabbit 2..............	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	4	4	3	3	3	—
Rabbit 1 before immunization......	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Rabbit 2 before immunization......	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

It is clear from the experiment shown in Table IV that while heating does not produce any change in the non-formalinized suspensions, formalinized *Rickettsiae* become agglutinable, which means that heating removes a factor that under ordinary conditions prevents agglutination in anti-*Proteus* serum.

The complementary experiment shown in Table V indicates that the formalin factor is not active in the same way when added to the *Rickettsiae* 24 hours after such suspensions have been made in saline. A test with human typhus serum was set up in order to control agglutinability by the homologous antibody.

We cannot at present explain the mechanism of the formalin action except that, as found for pneumococcus by Zinsser and Tamiya (3), formalin preserves from deterioration or disintegration some delicate antigenic element present in the *Rickettsia* body upon which agglutination in anti-*Proteus* serum depends. Heating, it seems reasonable to assume, removes a factor which masks the action of the serum in a manner analogous to that by which the type-specific carbohydrate factor masks species-specific agglutination in the pneumococcus group.

TABLE V

Serum dilutions	1:20	1:40	1:80	1:160	1:320	1:640	Mexican *Rickettsiae* formalinized 24 hrs. after washings in saline
Anti-*Proteus* serum	—	—	—	—	—	—	Unheated
Anti-*Proteus* serum	—	—	—	—	—	—	Heated
Human typhus serum		1		3		1	Unheated
Human typhus serum		1		2		1	Heated

TABLE VI

Absorption of Anti-Proteus Serum by Mexican Rickettsiae (Formalinized, Unheated)

	Proteus X-19					Heated formalinized *Rickettsiae*			
Serum dilutions	1:200	1:400	1:800	1:1,600	1:2,500	1:40	1:80	1:160	1:320
Anti-*Proteus* serum unabsorbed	4	4	3	2	±	1	2	1	—
Anti-*Proteus* serum absorbed	4	4	3	±	—	—	—	—	—

Since we had thus succeeded in obtaining agglutination of formalinized and heated *Rickettsia* suspensions in anti-*Proteus* serum, we proceeded next to study the absorption of such serum with *Rickettsiae.* We found that, although only the treated *Rickettsiae* were agglutinated, the results of absorption were the same whether or not this procedure was carried out with normal or with formalinized heated organisms. In the experiment shown in Table VI, the absorption was carried out with normal *Rickettsiae.*

From experiments like the one given in Table VI, it is clear that

agglutination, both in European and Mexican, established by Zinsser and Castaneda in a preceding communication.

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THE FORM AND FUNCTION OF SYNOVIAL CELLS IN TISSUE CULTURES

I. MORPHOLOGY OF THE CELLS UNDER VARYING CONDITIONS

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PLATES 4 TO 6

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The following investigations concerning the nature and function of the synovial tissue were stimulated by the current diversity and confusion of opinion on this subject. A study of an extensive literature revealed at least two problems requiring elucidation: one, the nature of the synovial membrane; the other, the source of the synovial fluid.

Only the essential opinions of the many workers in this field will be briefly reviewed here.[1] The older investigators (Winslow (1), von Kölliker (2) and others) regarded the synovia as a continuous epithelial membrane that secreted the articular fluid. According to Bichat (3) it was most closely related to the serosal membranes. His (4), especially, objected to considering it as epithelium and contended that it was endothelial and formed from the second embryonic layer. From extensive investigations Heuter (5) concluded that the synovial covering was a very cellular connective tissue membrane. Schweigger-Seidel (6), Landzert (7), Schneidemühl (8) and their contemporaries clung to the older opinions that it was either an epithelial or an endothelial membrane. Heuter's views, nevertheless, found confirmation in the contributions of Hagen-Torn (9), Braus (10) and especially in those of Hammar (11) who, from thorough histologic studies, designated the synovial cells as ordinary fixed connective tissue elements. Although this opinion subsequently attracted many exponents, still it is by no means generally accepted. Thus, both Soubbotine (12) and Mayeda (13) believe that the joint cavity is covered with genuine glandular epithelium, Aschoff (14) and Kaufmann (15), in their text-books, speak of an endothelium; and Lubosch (16) regards the synovia as a modified cartilaginous tissue. The diverse opinions concerning the mode of formation of the synovial fluid are reviewed in the subsequent communication (17).

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[1] A more extensive review of the literature is given in an article by Vaubel, Über das Synovialgewebe, in *Virchows Arch. path. Anat.*, in press.

From a review of the literature it is obviously difficult, if not impossible, to settle the moot points by means of simple histologic examinations; hence additional light was sought in tissue cultures from which the following questions seemed answerable.

1. What is the morphologic type of growth of the synovial cells and can it be differentiated from that of other cells of mesenchymal origin, especially from typical fibroblasts and covering cells of the serosal membranes?
2. Is it possible to detect the formation of mucin in tissue cultures?
3. What conclusion may be drawn from the results so obtained concerning the morphology and function of the synovial lining?

Answers to the first question are presented below, and those to the second are given in the subsequent paper (17).

EXPERIMENTAL

As original source of material the synovial tissue of rabbits was employed; usually that from different areas of the knee joint was selected as follows: the fat pad in the neighborhood of the patella which is covered with an especially thick layer of synovial cells, the tissue from the upper synovial recess at the side of the quadriceps tendon, occasionally a portion of the posterior part of the joint capsule. It was relatively easy to separate the synovial membrane from the underlying muscular or tendinous tissues, but in explants containing fat it was not possible to eliminate entirely the fat cells. Generally four pieces 1 to 2 mm. in diameter were explanted into each of a number of so called micro flasks or into Carrel D flasks; but before being placed in the flasks the tissue was washed for half an hour in Tyrode's solution in order to remove any adhering joint fluid. Often cover-glass preparations were also made. The culture medium consisted of heparinized plasma of normal rabbits in quantities of 0.5 cc. together with 0.25 cc. of a 10 per cent extract of rabbit's spleen. This extract was prepared by mincing the spleen very fine, suspending it in nine parts of Tyrode's solution, centrifuging at high speed and removing the cell-free supernatant fluid, which was designated as a 10 per cent extract.

Following the coagulation of the plasma, the contents of some of the flasks were washed twice, each time with 1 cc. of Tyrode's solution which was allowed to remain on the clot for half an hour, then pipetted off, in order to remove as well as possible growth-stimulating substances contained in the splenic extract. Finally, 0.25 cc. of rabbit plasma was added, the flasks tightly stoppered and placed in the incubator. The same procedure was repeated with all flasks after a period of 4 to 5 days: the contents of the flasks were washed with Tyrode's solution, then fresh plasma and splenic extract added (so called patching) and into a part of the flasks 0.25 cc. of plasma was again placed following the washing with Tyrode's solution. After about 10 days each growth was divided into four or more pieces and transplanted into new flasks containing medium similar to that described above. These transplants were washed, patched and nourished the same as the explants. The washing of the cultures had the object of holding the nutritive conditions at a low level, for, according to Fischer and Parker (18), the nature and function of the original tissue is better maintained and its tendency to change into fibroblasts is diminished when cells are grown in media of low nutritive value.

Each day the cultures were examined microscopically and in certain instances the changes occurring during definite intervals were recorded by drawings made with the aid of a camera lucida. Suitable living and fixed cultures were also photographed.

For histologic preparations the cultures were excised from the plasma clot and fixed in formol, Zenker, Zenker-formol or 2 per cent osmic acid; then embedded in paraffin, and sections cut therefrom were stained with hematoxylin and eosin, Van Gieson, eosin and methylene blue, toluidine blue and mucicarmine. For low power examination of a complete culture the material in a flask was fixed in formol or Zenker and stained with hematoxylin.

Altogether the synovial tissue from six rabbits was explanted, and the number of cultures (explants and transplants) studied amounted to about 400. In addition, the following controls grown under the same conditions were employed: fibroblasts from the heart, testis, subcutaneous tissue and muscle; serosa from the peritoneum, pleura, pericardium and tunica vaginalis.

Morphologic Findings

Fibrinolysis and Character of Growth in Liquid Medium.—In fresh explants the individual tissue elements were naturally difficult to recognize, because it was impossible to separate sharply the synovial membrane from the underlying structures, and the presence of numerous fat cells introduced certain optical difficulties. Nevertheless, one could recognize here and there at the margins of explants the richly cellular structure of the articular lining membrane, projecting beyond the zone of the fatty tissue and clearly differentiated from the capsular connective tissue because of a much smaller number of fibrillae. Fibroblasts were not recognizable, and capillaries were only rarely seen. In so far as they could be distinguished, the cells of the synovial membrane appeared large, round, or polygonal and close together.

After 4 or 5 hours certain alterations occurred in the plasma coagulum, especially in zones where the synovial membrane was most easily seen. The fine fibrillar meshwork of fibrin became indistinct and vanished; these small zones of liquefaction then slowly increased, and simultaneously there was an emigration into the plasma of leucocytes, lymphocytes and monocytes, which were easily differentiated from the larger cells that appeared later. These smaller cells were few in number and did not proliferate to any great extent.

After 24 hours cellular proliferation was detectable in some of the explants; short and plump cytoplasmic processes projected outwards into the liquefied plasma, and somewhat longer processes extended into the solid clot. The outwandering of these cells was somewhat difficult to follow during the first 3 days after explantation. The larger cell bodies quickly followed the cellular processes into the area of fibrinolysis, most of them separated themselves from the original piece, became rounded and dropped into the fluid zone and subsequently became attached to its walls. Cells constantly proliferating from the explant passed into the fluid until the liquefied area often appeared covered with cells. Thence they passed into the meshwork of the clot, often as round forms which showed a capacity for extensive migration; for after 4 or 5 days they were not infrequently seen at least 1 cm. from the explant. These emigrated cells would then multiply and give rise to new islets of growth.

In the fibrinolytic zones the cells often formed a network by sending out processes which joined with those of their neighbors. Cells round in the beginning became spindle-shaped by forming two or more processes; after a time they broadened and showed a sharp nucleus outlined against a heavily granular cytoplasm. Additional cytoplasmic projections appeared, giving the cells a star shape, and joined with cells lying in the coagulated plasma. In places where the liquefaction involved the entire thickness of the medium the emigration of cells was more extensive (Fig. 4); here an extension of one or two short processes was followed by a larger elongated cell body with sharply outlined nucleus. The young cell remained joined to the explant for a time, then detached itself and moved away. New cells pressed out and arranged themselves close to one another in round or polygonal shapes, thus forming a mosaic that covered the bottom of the cavity in the clot. The cells apparently multiplied readily and often remained connected with one another by means of filiform projections. Singly or in groups they migrated with an ameboid motion and a constantly changing outline. New cellular islands arose forming areas of organization, with epithelioid cells at their centers, elongated cells at their margins, and with cytoplasmic bridges uniting the different elements. Thus, cells by sinking to the bottom of the liquefied zones, then proliferating and joining with others, often formed a membranous tissue that early contained a few defects; but when these were closed the appearance was that of an epithelioid structure (Figs. 10, 12, 13). A hole in the plasma occasionally decreased in size or disappeared entirely; this was apparently induced by a shortening of the cellular processes which pulled the cells together, and consequently closed the cavity. Cells from the liquefied zone sometimes joined with those in the solid medium in the form of strings of pearls (Fig. 14). With a decrease in the size of the cavities the cells became more crowded and formed two or more layers until finally after 7 or 8 days the defect was often entirely closed by a dense meshwork of cells intimately united by numerous intercellular bridges.

Such a healing of defects was not always the rule; at times the entire plasma coagulum became liquefied by a gradual increase in the several zones of fibrinolysis until the explant became merely an island of tissue either attached to the wall of the liquefied zone or free in the fluid. Under these circumstances the cells lining the cavity in the clot became stretched and formed a narrow concentric meshwork at its periphery. The lengthening cellular prolongations seemed to be under tension and stretched by a constant enlargement of the hole, for occasionally it was possible to observe a sudden breaking and retraction of these intercellular bridges, much as though an overstretched rubber band had been broken (Figs. 3 and 8). Groups of cells thus detached assumed an epithelioid appearance and became new centers of organization for growths. Generally, however, with a constantly increasing fibrinolysis, the number of cells decreased and the bits of newly formed tissue became smaller. If, on the other hand, the culture was patched by adding new plasma and splenic extract, new life seemed to be imparted to the cells, which started to multiply both in the original explant and in the islets. In a short time an extensive growth resulted which, not infrequently, again induced lysis of the patching clot.

When a thin layer of fibrin remained in the bottom of a cavity in the clot and next to the glass wall of the flask, the growth from the edge of the explant was much more intense. The emigrating cells were not so flat, but remained spindle-shaped with one or two short projections directed in the line of growth and with a long basal thread. The course was similar to that previously described; by rapid division the cells became closely layered, like epithelium, but with many cytoplasmic bridges. Again due to continued fibrinolysis, the cellular masses would be drawn together and assume the appearance of an epithelial-like synovial membrane. Sometimes the elongated cells were forced apart and became rounded, isolated cells that either sank to the bottom of the fluid or attached themselves to the walls of the liquefied zone. If there occurred complete liquefaction of the thin fibrinous scaffold supporting an extensive growth of new cells the intercellular bridges became overstretched and broken, and the new growth was either pulled towards the old explant or formed new centers at the periphery of the zone of fibrinolysis.

About synovial villi striking phenomena occurred: fibrinolysis was marked and there was early emigration of large round cells (Fig. 3). Following the formation of many filiform cytoplasmic processes into the edge of the liquefied plasma, the original synovial cells appeared to wander into the area of new growth with a gradual thinning of the explanted tissue. The fatty cells in the middle of the villus moved out, and subsequently remained unaltered. After a week the defect in the plasma was organized and the structure of the villus was no longer recognizable.

The marked solution of the plasma clot above noted is doubtless comparable with that described in cultures of intestinal epithelium (Fischer (19)), of mesothelium (Herzog and Schopper (20) and our own observation), and in pancreatic tissue (Kapel (21)). It is probably to be sharply differentiated from that observed in tissue cultures of dying cells and of certain tumors. A normally growing culture of fibroblasts has the capacity of dissolving fibrin, but only to a slight degree. Recently Chlopin (22) noted that the gradual digestion of the fibrin in the midst of the zone of proliferating cells corresponded with the extent of their growth. With suitable staining he usually found no residue of the fibrin in the thick areas of growth of young cells; hence he concluded that the cells not only invaded the fibrin scaffold, but gradually dissolved it. I have repeatedly observed that this tendency is especially marked when the culture has too little nourishment. Cultures of fibroblasts grown in plasma clots which had been deprived of much of their nutritive properties by washing, gradually digested the fibrin, so that eventually one or more little cavities appeared. That death of the tissue, however, was not responsible for the fibrinolysis was made evident by the fact that patching of these cultures with fresh plasma and extract was followed by a renewed growth without new cavity formation. One must, indeed, differentiate the fibrinolysis arising from dying and degenerating cells. Fischer (19) showed that this condition could be carried over to healthy cultures by treating them with Berkefeld filtrates of the fluid from degenerating cultures; probably the enzymes were set free from the dying cells and were not living agents. It is a matter for discussion how far the proteolytic ferments derived from cultures of tumor cells are dependent upon death of cells and how much upon a virus-like living agent; as for example, in the lytic substances derived from a Rous sarcoma.

It seems possible to differentiate at least three different factors leading to fibrinolysis: (1) a physiological ferment excreted by the cells and also detectable in the body; (2) newly developed ferments arising from peculiar conditions in the tissue cultures; (3) ferments liberated by death of cells. Fibrinolysis from tumor cells may occupy still another category. One cannot state definitely whether these different ferments are identical chemically and physiologically. The occurrence of zones of clearing in cultures of synovial cells is probably attributable to normally occurring ferments, which are also demonstrable as a part of the physiological functions of the synovial fluid.

Growth in Plasma Coagulum

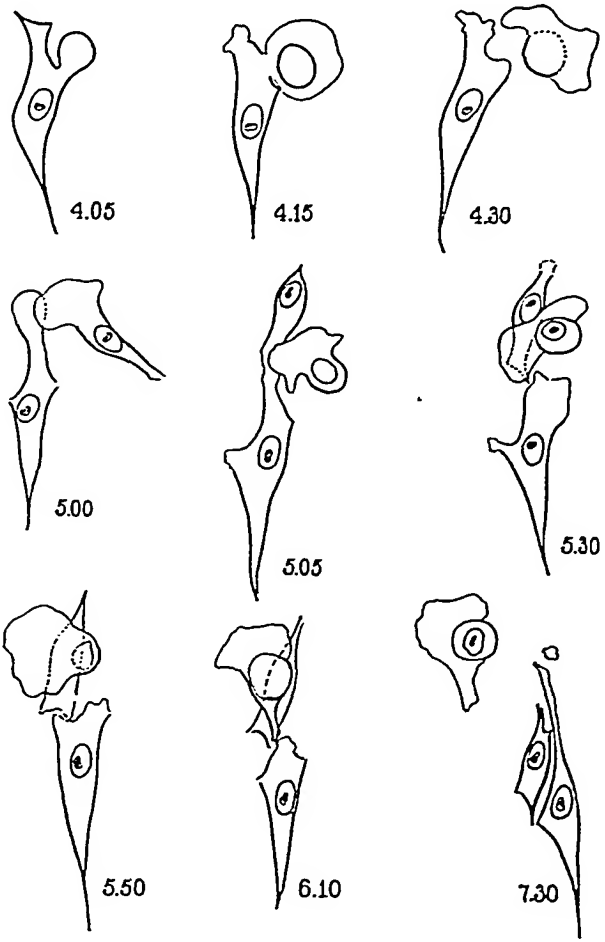
The growth of synovial cells in liquefied medium has been presented first because it seems to have special significance in the elucidation of certain conditions in the joints. Fibrinolysis was most marked where explanted synovial cells were most numerous. This type of growth was by no means the general rule; it occurred most readily when small amounts of tissue extract were present in the medium. Solution of the fibrin was less marked near portions of explants containing fewer synovial cells, and the emigration and type of growth was somewhat different. Instead of the early round forms previously described, there appeared more spindle- or tulip-shaped cells in the solid plasma. The plump cell bodies usually showed two long cell processes; in many cases the long basal cytoplasmic thread formed a bond of union with the next cell, hence they resembled a tulip in contour. At times these migrating cells separated themselves from the explant by a breaking of the protoplasmic bridges, and thus they existed free in the surrounding medium. The long axes of these cells were not as a rule arranged radially but were often parallel to the edge of the growth.

After 48 hours one could see a spongework of cells, mostly isolated but regularly arranged about the explant, and joined by cytoplasmic bridges. The transitional character of the cell outlines was most striking; those lying near one another appeared to alter their shapes; processes were extended and withdrawn; a cell would broaden in one place and apparently flow into the surrounding medium with a resulting indistinctness of the cellular outline.

After about 72 hours there was a loose network of cells often without any clear arrangement of its component parts. Filiform cellular processes were often astonishingly long, and extended from cell to cell (Figs. 5, 6, 11). Such intercellular bridges were frequently the result of an incomplete division of the cytoplasm when the cells divided; but instances were observed where they were formed secondarily. That we were dealing here with a genuine syncytial union of cells is shown by the fact that there was a free movement of granules from one cell to another. The existence of true cellular anastomosis has been denied by Lewis (23), who regarded the syncytial junctions as simple adhesions. Kredel (24) and de Garis (25) assumed the same attitude because they could detect no transfer of granules among neighboring cells. Laser (26), Levi (27) and Fischer (19), on the other hand, described a distinct migration of dyestuff, mitochondria and granules from one cell to another. The fact that intercellular processes arise and withdraw is no ground for regarding them as simple non-communicating structures.

The primary intercellular bands appeared often to result from amitotic cell division, a condition repeatedly observed. Portions of the nucleus were pinched off without any essential alteration in the structure of the chromatin; and the cellular outline was not necessarily affected. Two, and occasionally three, nuclei were sometimes found in a single cell; then after a time the cell would become elongated, the two nuclei would separate, one would wander into a cellular prolongation that simultaneously became swollen as a result of a flowing into it of cytoplasm. The resulting club-shaped portion of the cell advanced still further, the union with the other cell half became smaller and on the fore end of the new cell appeared one or two new cellular projections. This process could be repeated several times without breaking of the cellular unions (Fig. 9). Thus rows of cells appeared, sometimes branched, that extended far out into the medium because of their rapid division; often they also retained their tulip form.

The question of amitotic cellular and nuclear division is still much discussed. Kreibisch (28) described direct division in cultures of epithelium; Holmes (29) and Lewis and Webster (30) saw nuclear amitosis without subsequent cellular division. Schopper (31) objected to an unreserved description of amitosis on the ground of the difficulty of obtaining a clear picture in multiple layers of cells. In a recent communication Parker (32) clearly described numerous amitotic divisions during the transformation of fibroblasts into macrophages. I can fully confirm his observations in the division of synovial cells.



TEXT-FIG. 1. Camera lucida drawings of a dividing synovial cell during a period of 3½ hours. Time drawings were made indicated by the figures.

In order to record accurately this process camera lucida drawings were made, that show the formation of new cells from a mother cell in the interval of 3½ hours (Text-fig. 1). The first drawing (4.05) represents the beginning of a budding; a picture of the original typically outlined cell was not available. At first a round cell with a clearly discernible membrane separated itself, and retained during the entire observation period its so called macrophage form. Then (5.05) there arose in another place a second cell body that soon broadened out and lay near the mother cell. In the last drawing (7.30) there is a small body that apparently arose from a "plasmarest," and, in so far as could be determined, had no union with any cell. In spite of the fact that detailed nuclear division could not be observed, the new formation of cells can only be explained by amitosis.

Mitotic cellular division, on the other hand, was more frequently observed, and was especially clearly seen in stained preparations. The cells appeared in general to be rounded, were highly refractive and showed indistinct nuclei. Cellular division occupied a relatively short time; and the daughter cells assumed the typical form.

After 4 or 5 days a thick network of cells had formed in which the individual elements were united by syncytial communications. At the border of the growth, however, there were many isolated cells of the same type in which ameboid alterations in shape and movement were easily seen. Many of these cells advanced further into the plasma, built a branching network and thus formed an irregular border to the growth.

The foregoing description refers to the growth in the midst of the plasma clots. On the surface of the medium and next to the glass wall of the flasks certain differences were seen. In such places there was a marked tendency to membrane formation very similar to that described in fibrinolytic cavities in the medium. The cells on the surface of the medium were flattened and hence the nucleus and coarse granular structure of the cytoplasm were more visible. The appearance of the growths was essentially the same as that already pictured. "Tulip cells" with one basal and two advancing cell processes were numerous. Individual cells or groups bound together were often seen distinct from the main growth; these formed new centers which grew, fused with one another and eventually formed a large membrane.

The three half schematic drawings of a single area of growth made with the help of a camera lucida give a good idea of the development of such a membrane (see Figs. 18–20). They show the development of a second subculture of apparently pure growth of synovial cells.

Only the growth next to the glass was drawn, while that in the midst of the medium was disregarded. Fig. 18 shows the growth about 72 hours after transplantation. Individual cells were parallel to one another; some were connected by filiform cytoplasmic processes often stretched over considerable distance. Some cells were seen in different stages of amitosis. A rounded cell in amitotic division is indicated at *x*. The picture resembled somewhat a so called typical fibroblastic growth, but the cells were larger, more plastic, and had coarser granulation. Subsequent growth showed greater differences. 1 day later (Fig. 19) the cellular processes were distinctly more numerous, the cells more contracted and star-shaped; the intercellular bands were sometimes formed secondarily by junction of processes from neighboring cells and sometimes primarily by persistence of cytoplasmic bridges formed at the time of the cellular division. Elsewhere in the plasma clot numerous cavities had appeared.

Fig. 20 was drawn from the culture 5 days after transplantation; but on the previous day it had been washed and nourished with fresh plasma. This imparted a renewed growth impulse. The holes were now smaller and the number of intercellular bands greater so that one could correctly speak of a syncytial membrane that in places showed an epithelial character. The arrangement of the cells was irregular throughout and the size of the single elements varied greatly. The intercellular processes were often extremely thin and delicate, yet sharply delimited. Many of the cells were united by numerous cytoplasmic bridges, often parallel to one another. The picture reminded one of bits of semifluid gum pulled apart. The cell membrane thus formed could thicken and form a mosaic as pictured in Figs. 12 and 13.

Mixed and Pure Cultures; Transformation to Fibroblast Types

It seems necessary to discuss briefly the other cell types present in the original explants: fibroblasts, endothelial cells and fat cells. The fat cells underwent neither proliferation nor other essential change.

The development of fibroblasts and vascular endothelial cells was apparently much influenced by the number of synovial cells and their mode of growth. There was evidently an inverse ratio in the extent of the growth of synovial cells and fibroblasts. With vigorous growth of the synovial cells the endothelial and fibroblastic growth was scanty, and where the latter predominated in the same culture synovial cells were often absent. This naturally depended somewhat upon the distribution of the different cell types in the original explant; for fibroblasts seemed to be inhibited by synovial cells on account of the fibrinolytic ferments elaborated by them. Synovial cells, moreover, grew out of the explants many hours ahead of the fibroblasts and endothelial cells and thus had a chance to exert their inhibitory influence.

Mixed cultures were seen containing festoons of synovial cells, some isolated and some in a meshwork, arranged around a center of fibroblasts and endothelial cells. The larger ameboid synovial cells with large nuclei and coarse highly refractive granules appeared exceedingly plastic, with their long axes pointing in all directions. The fibroblasts were arranged radially, were close together, spindle- or needle-shaped, and showed a fine granulation. The nuclei were oval and not so sharply delimited. On the surface of the medium their shapes were more irregular, due to the more numerous cellular processes; but tulip forms, so characteristic of synovial cells, were seldom seen.

Vascular endothelial cells grew typically from the first as membranes or tongues of relatively transparent cells with regularly arranged elements forming distinct designs.

Upon further growth the mixed character of the culture sometimes persisted; again one or another cell type was suppressed. Often the advantages of the synovial cells over the other cell types were apparently lost, especially when fibrinolysis was marked. In general, however, the rapid, predominating growth of the synovial cells made it relatively easy to obtain them in pure cultures. Especially favorable for the purpose were those areas showing a moderate sized zone of liquefaction. After 10 days, growth was sufficiently thick and broad to permit one to excise pieces containing none of the original explant. Thus, it was possible by proper selection and transplantation every 10 days, to obtain a typical growth of synovial cells practically free from any other cell types, especially if the nutritive conditions were maintained at a relatively low level and the rate of growth thereby somewhat suppressed.

Later, however, it was often not possible to prevent a transformation into a growth having a fibroblastic character. The cells became small and spindle-shaped with numerous long, sharp, fine processes, and contained smaller and less clearly demarked nuclei. The typical coarse and highly refractive granules of the synovial cells became smaller and less distinct, and were no longer seen in the next transplant. Zones of fibrinolysis no longer appeared. From such a transformed culture it was only rarely possible to revive the synovial type of cells in subsequent subcultures. Generally when a fibroblastic character appeared it remained even though the method of Fischer and Parker (18) was applied in an attempt to induce a reversion.

Some of the cultures, nevertheless, retained the typical synovial cell characteristics even after the sixth transplantation, *i.e.* over 60 days; others changed their type after a few passages. They assumed all the characteristics of fibroblasts, or the rate of growth lagged somewhat, and cell division became less frequent. The individual cells enlarged markedly, contained one or more nuclei and threw out numerous cell processes (Figs. 11, 16). Coarse granulation was marked in some cells, while in others it was absent. A tendency to fibrinolysis remained in these cultures. Such forms could remain stable in the next passage, and tended to recur when the number of cells in the transplant was small or the transplant minute. Cultures having this cell form showed a marked tendency to fatty degeneration.

One cannot state definitely all the conditions responsible for the tendency of the cells to undergo transformation in type; the nutritional conditions doubtless played a major rôle, for with overnourishment synovial cells seemed specially liable to change into fibroblasts. With undernourishment, induced by long washing of the cultures and failing to add either plasma or splenic extract, the cells remained distinctly smaller, quickly showed signs of degeneration and could not be distinguished from fibroblasts grown under the same poor conditions.

It would lead too far afield to undertake an extensive discussion of the differentiation and dedifferentiation of tissue in cultures. According to recent opinions (Fischer and Parker (18)) it is impossible to identify the origin of different tissues from the morphology of cells alone. Apparently all cells of mesenchymal origin possess the capacity of reverting to a certain type currently designated as fibroblastic. This occurrence under conditions usually existing in ordinary tissue cultures has given rise to the idea that all mesenchymal tissues easily become transformed in the cultures to fibroblasts with a loss of the special functions they have in the intact and healthy body. That this transformation is dependent upon peculiar cultural conditions is evident from the work of Fischer and Parker, who, with suitable methods, could induce the production of cartilaginous ground substance and characteristic cell forms in cultures of chondroblasts. With a similar technique it was possible to determine the physiological differences between connective tissue stocks of varying origin. It is probable that with the development of suitable methods it will be possible to supply each with proper cultural conditions so that it will develop and retain its chief physiologic function.

Round Cell Formation

Noteworthy are some further observations having to do with the occurrence of many smaller round cells, for it was possible to follow their development in both explants and transplants. They appeared to arise exclusively from long cells resembling fibroblasts in media containing either large amounts of splenic extracts or in those composed almost entirely of plasma.

In certain cultures there appeared about the 3rd or 4th day a number of smaller and markedly ameboid round cells, which wandered far out into the medium. Their derivation from the synovial cells was easily followed. A round bud-like swelling appeared in the region of a thin cell prolongation, and after a time separated itself from the mother cell as a small round element, containing a nucleus decidedly smaller than that of the original cell, as was best demonstrated in stained specimens (Fig. 17). These small cells arose from amitotic division of the nucleus of the mother cells, which were then pinched off with a small amount of cytoplasm, while the original cell retained its form and often underwent a similar second division; but occasionally it became rounded and wandered through the medium. The budding sometimes occurred so rapidly that the cytoplasmic bridges were not ruptured when the next cell was formed, and thus there appeared a picture like a string of pearls (Fig. 14).

These small round cells divided repeatedly, apparently by mitosis; but occasionally amitotic cell division was observed. Because of the rapid division round cells of all sizes appeared, some so small that it was difficult to detect any cytoplasm even in stained preparations. In the course of a few hours or a day these cells increased in size, broadened out and soon showed a marked granulation of the cytoplasm. By further division they formed little groups of cells, often assuming a spindle shape and joined by intercellular processes. After 1 or 2 days new islets of tissue were thus formed that could not be distinguished from the original either in cell type or growth type.

One might compare these round cells with the macrophages, which according to some authors, arise from fibroblasts. Their manner of derivation corresponds in principle to the conditions frequently observed by me in amitotic cell division, yet here there occurred an unusually rapid and stormy course of events. Thus, because the original cell was unable to attain its normal size after division, the daughter cell contained only a small nucleus and little cytoplasm; hence it was decidedly smaller than the mother cell. No cause could be found for this interesting phenomenon, but it is noteworthy that only rarely did all of the cultures in the same flask give rise to it simultaneously.

The development of fibroblasts from macrophages has been often described (Carrel and Ebeling (33), Fischer (34), Maximow (35), Bloom (36), Chlopin (37) and others). The reverse condition has been much discussed, and by some denied on theoretical grounds. Carrel and Ebeling have observed such a transformation at least twice; likewise Fischer (38), Fischer and Laser (39), Ephrussi and Hugues (40), W. and M. von Möllendorff (41), Schopper (42), Börner and Herzog (43), and Herzog and Schopper (20); while Tannenberg (44) denies the formation of macrophages from fibroblasts and contends that these forms are merely degenerated fibroblasts. Especially interesting in this connection are the observations of Parker (32), who by a simple alteration in the nutritive state of the medium and without the introduction of any toxic substances, was able to bring about a transformation of connective tissue elements into macrophages.

From repeated observations like those recorded above I could determine that at least a large part of the macrophage-like cells originated from fibroblast-like synovial cells by amitotic division; and that these cells could further divide by both amitotic and mitotic cell division. The von Möllendorffs describe amitosis, and believe it may be expected especially frequently in macrophages. Parker, in discussing amitosis, says that there is no ground for doubting its occurrence or for resorting to the simple theory of cellular degeneration to explain the origin of the round macrophages.

Noteworthy also was the formation of new islets of growth by round macrophage-like cells which had separated from the original piece and wandered out into the medium. These islets often gave rise to new tissues large enough to be easily seen macroscopically; they were sometimes at least 1 cm. from the original piece, without any detectable intercellular bridges connecting the two. Hence I am inclined to qualify the statement of Schopper and Fischer that it is impossible for an isolated cell or for a small group of cells to multiply in cultures under these conditions. In the events here described the isolated cells had not been brought into fresh medium. They were probably not genuine newly formed macrophages, as in Schopper's experiments, but were specially active rounded forms of synovial cells, that were possibly able to induce certain peculiar changes in the medium because of their elaboration of a fibrinolytic ferment.

Stained Sections and Fresh Preparations. Granulations and Supravital Staining

The findings with stained sections corresponded in general with those made on living cultures; hence only the additional points brought out by staining are presented. Of the different fixatives employed 2 per cent osmic acid was the best, for although formol and Zenker fixation gave useful pictures the cellular structures and granulation were not nearly so well preserved. For examination of fresh material the synovial membranes or the cultures excised from the plasma were carefully spread out on a slide, moistened with Tyrode's solution and covered with a cover-glass, the edges of which were sealed with vaseline; then an oil immersion lens could be employed for observations.

The structural correspondence of the synovial membrane with that seen in the tissue cultures was often striking, not only in fresh unfixed preparations but also in stained sections. In sections of the cultures all of the above described types and arrangements of cells were found. Where fibrinolysis had occurred there was often a membrane composed of one or more layers of typical cells, having cellular processes which at times were seen only in the stained specimens; branched or rounded cells, isolated or in groups, were also present. So called "capsulated cells" were sometimes seen, that is cells surrounded by clear zones; but it is doubtful whether we were dealing with a true capsule as in the case of chondroblasts. Probably the appearance was due to a liquefaction of the plasma, induced by a fibrinolytic ferment, or it may have followed a simple retraction of the cells from the medium as a result of the fixation; on the other hand, similar pictures were not seen in the controls composed of other tissues.

Especially noteworthy and peculiar to the synovial preparations was the heavy granulation of the cells. In fresh tissues almost all of them contained coarse granules extending out into the cellular processes (Figs. 2, 5, 6). These granules varied in number and size in different cells, and some cells showed only fine dust-like granulations. The cytoplasm had a reticulated structure. The nuclei were well formed and usually not covered with granules; and near them fine centrospheres were sometimes seen. In this respect cultures and vital preparations corresponded, as they did in the appearance of granules. The latter appeared to increase in size and number with the age of the culture, and apparently larger globules were produced by a fusion of smaller ones. Osmic acid fixation demonstrated that most of the granules were not due to fatty degeneration of the cytoplasm, even though some synovial cells, and especially those near fatty tissue in the explant, contained black fatty droplets.[2]

All the cells contained, in addition, numerous colorless granules exceeding in size and number the fatty globules and not demonstrable in Zenker- or formol-fixed sections stained with hematoxylin.

In applying the mucin-staining toluidine blue, according to the method devised by Metzner (45) and used by Mayeda (13) and Kling (46), it was found impossible to stain regularly the granules in all of the cells in any one preparation. Occasionally, however, in both explants and transplants, the newly grown cells showed many greenish blue to bluish granules, often with a distinct reddish metachromatic tinge. According to Groebbels (47), the bluish granules consist of premucin and the reddish ones of mucin. Often, however, there were unstained granules in the same cells. No staining was obtained with mucicarmine and muchematein.

Supravital staining with neutral red yielded especially interesting results. Both cultures and fresh tissue were treated similarly; *viz.*, mounted on a slide previously coated with a layer of the dye (Sabin (48)). Some cultures were stained by filling the culture-containing flask with a 1 to 5,000 solution of the dye in Tyrode's solution. The flasks were then stoppered and observed directly under the microscope or were allowed to stand a certain time after which the cultures were removed and mounted on slides. Occasionally the knee joint of a moribund rabbit was filled with a solution of the dye; 30 minutes later, following the death of the animal, the synovia was excised, mounted on slides and examined. Joints thus treated were distinctly red, especially in those places containing the most synovial cells.

Quickly taking up the dye, many of the cells of the articular membrane and many of those in the tissue cultures soon appeared to be laden with red globular granules. Nucleus and cell cytoplasm remained colorless for about 2 hours when cell death ensued. Rounded ameboid cells with red granules and colorless cytoplasm could hardly

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[2] Such a tendency for the synovial cells to take up fat is discussed by Lubosch (16).

be distinguished from clasmatocytes. Both the nucleus and cyt plasm of degenerating or dead cells immediately stained diffusely re Some cells were seen with only a portion of the granules stained, ar occasionally none took up the dye. While ultimately both fres synovia and cultures stained similarly, the dye penetrated into tl latter more slowly because of the thick surrounding plasma. Tl synovial membranes of rats, guinea pigs and monkeys, vitally stainc in the same manner, took the dye as did that of the rabbits.

Nagel (49) and Chlopin (22), in contradistinction to Lewis (50) and von Mölle dorff, think that the mechanism of supravital absorption of basic dyes is depende not only upon a staining of lifeless cell inclusions, but is also due to an active phys ological process closely related to secretion. Noteworthy is the statement of Fi chel (51) concerning the Leydig cells in the cutaneous epithelium of salamand larvae that stain intensely with all vital dyes. According to this author, one dea here with mucin granules avid for the dye. Possibly the neutral red-absorbin qualities of the synovial cells is due to a similar content of mucinoid fraction However, because of the failure of these intracellular granules to stain with muc carmine or muchematein, and also because epithelial mucin-forming cells do nc stain with neutral red *in vivo* (Bensley (52)), one cannot state definitely that th mucin elaborated by the synovial cells is present in them in the form of granules c globules.

Controls

While it is superfluous to describe fully the results with control cul tures, consisting of fibroblasts and serosal cells arising from variou sources, because these have been fully considered in the literature still certain differences from synovial cell cultures are deemed note worthy. Serosal cells grown from the various body cavities wer essentially the same, with some individual differences not to be dis cussed now. Serosal cells, in contradistinction to synovial cells, grew in close apposition one with the other, thus forming continuous mem branes that advanced from the edge of the explant into the medium Early the single cells were somewhat irregular in outline, but they quickly assumed an epithelioid appearance; several united and formed fingers which extended outwards and laterally; the sides of the fingers often fused in places and formed sheets enclosing clear cell-free zones. Such an outcome was most frequent with cultures derived from the omentum. Apparently the spaces in the membranes arose in this manner rather than from a lysis of cells. Such defects in the growths showed a great similarity to spaces in the omentum often mentioned in the literature (Maximow (35), Schopper (20)).

At the borders of the growths one found often large isolated cells having an ameboid motion; these apparently could separate themselves from the growth and later reattach themselves to it. Cytoplasmic processes were only visible at the edge of the growth, and were not seen in the midst of the membrane. Because in rate of growth the serosal cells outdistanced the fibroblasts it was fairly easy to obtain pure cultures of transplants by excising the proper areas from the explants; these retained the characters described above.

Occasionally zones of liquefaction appeared early at the edge of the explants of serosal cells as they did in the synovial cultures, but such areas were always smaller. The serosal cells could also separate themselves from the membrane, become rounded and migrate through the liquefied plasma and attach themselves to the borders of the cavities. They soon advanced into the solid plasma with an ameboid motion, and, either by division or by aggregating with other cells, formed islands from which new membranes of mosaic-like cells were fashioned. Secondary areas of fibrinolysis were not seen in explants or transplants. Except for variations arising from a different technique, these results agree with Schopper's description of serosal cells (42). Neither the blue nor red metachromatic staining of mucin granules was obtained either in serosal cells or fibroblasts stained with toluidine blue. The granules were always smaller than those of synovial cells. Similar differences in size were also obtained with supravital staining with neutral red; the granules were smaller and often absent from the serosal cells.

SUMMARY

A detailed description is given of the various forms assumed by synovial cells in tissue cultures under varying conditions. A discussion of correlation between different morphologic appearances and certain functions of the cells is deferred until the end of the next paper (17).

(References to both papers are given at the end of the second paper (page 94).)

EXPLANATION OF PLATES

Figs. 1 to 11 and 14 to 16 are photographs of living cultures. Figs. 12, 13 and 17 are of fixed and stained cultures. Figs. 18, 19 and 20 are drawings made with the aid of a camera lucida; they were all from the same area of culture observed for several days.

PLATE 4

FIG. 1. 72 hour explant. Formation of zone of liquefaction; dropping off of round cell forms into the fluid; filiform protoplasmal bridge; unusual number of round and spindle-shaped synovial cells are advancing into the plasma coagulum; many isolated cells. ×170.

FIG. 2. 72 hour explant. Detail from Fig. 1. Emigration of rounded synovial cells into the fluid zone. Cytoplasmic granulations clearly seen. ×420.

FIG. 3. 72 hour explant. Liquefaction zone in the neighborhood of a small articular villus. Note flattened cells on border of this zone. ×125.

FIG. 4. 72 hour explant. Beginning organization of cells in the bottom of a zone of liquefaction. ×125.

FIG. 5. 72 hour explant. Growth zone in solid coagulum, round and spindle forms; large round forms especially notable near the explant. ×170.

FIG. 6. 72 hour explant. Detail from Fig. 5 showing cytoplasmic granulation. ×420.

FIG. 7. 72 hour explant. Secondary fibrinolysis. Triangular and stellate cells in liquid (out of focus). ×125.

PLATE 5

FIG. 8. 72 hour explant. Zone of liquefaction with numerous round cell forms; thin intercellular bridges formed of filiform cellular processes; these are growing out from the original piece. ×125.

FIG. 9. 72 hour explant. Growth zone of the same culture as Fig. 8. A few round forms in the periphery with transition into spindle-shaped cells; multinuclear cells with numerous processes and intercellular filaments. ×125.

FIG. 10. Explant 6 days old, not renourished. Edge of the growth zone showing cells very large and heavily granulated, in places arranged like epithelium; small areas of beginning fibrinolysis. ×125.

FIG. 11. 12 day old second transplant. Culture washed three times and nourished with plasma once; very large cell forms with numerous long cytoplasmic processes. ×160.

FIG. 12. 120 hour growth of a second transplant; washed with Tyrode's solution after 72 hours; formol fixation, stained with methylene blue; epithelial-like membrane at the bottom of a fluid zone. ×115.

FIG. 13. Detail from Fig. 12. ×335.

PLATE 6

FIG. 14. 96 hour second transplant. Fibrinolysis in a very cellular area, epithelial-like arrangement of cells lying close together; cell bridges connecting this area with the periphery. ×125.

FIG. 15. 96 hour second transplant. Thin cytoplasmic processes stretched across a zone of fibrinolysis. ×125.

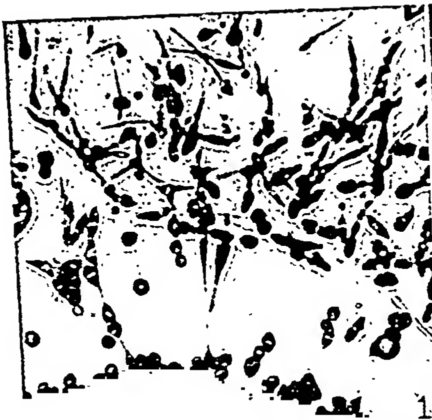
FIG. 16. The same culture as Fig. 11. Border of a zone of liquefaction. A group of round cells in the liquid; beginning of broadening and formation of cellular processes. ×160.

FIG. 17. 72 hour first transplant. Cover-glass culture. Formol fixation; stained with hematoxylin. Formation of small macrophage-like cells from spindle-shaped synovial cells. ×115.

FIG. 18. Drawing of 72 hour transplant. Syncytium composed mostly of elongated cells with relatively few intercellular bridges. At x a round form in amitotic division.

FIG. 19. Drawing of same area 24 hours later. Cells broader, and have more intercellular bridges.

FIG. 20. Drawing of same area 24 hours later. Culture had been renourished. True epithelioid membrane made up of dense syncytium.



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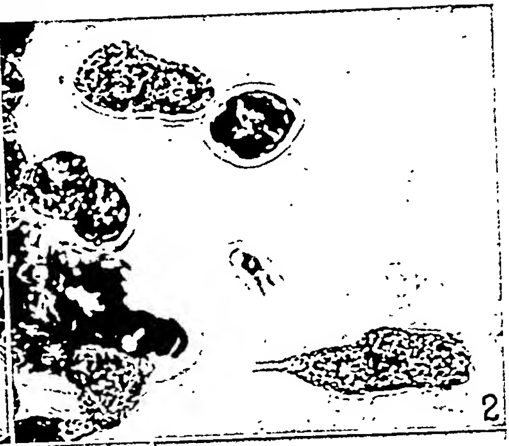

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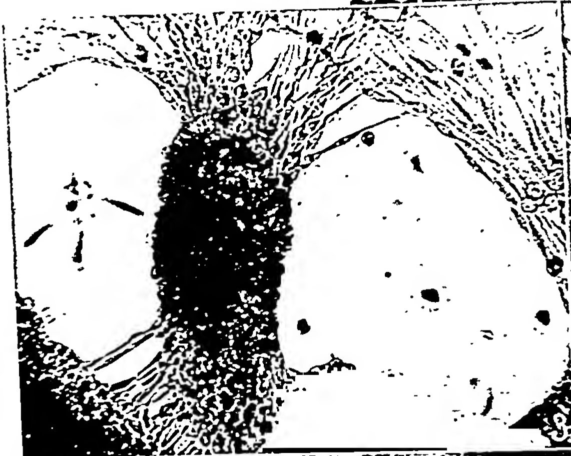

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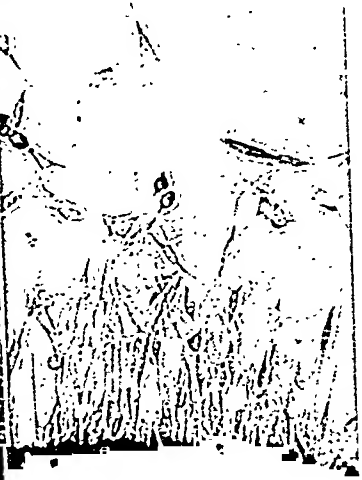

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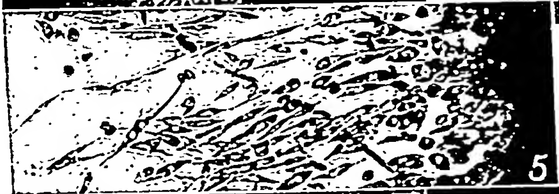

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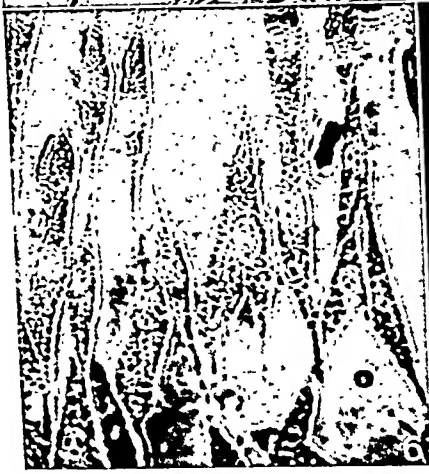

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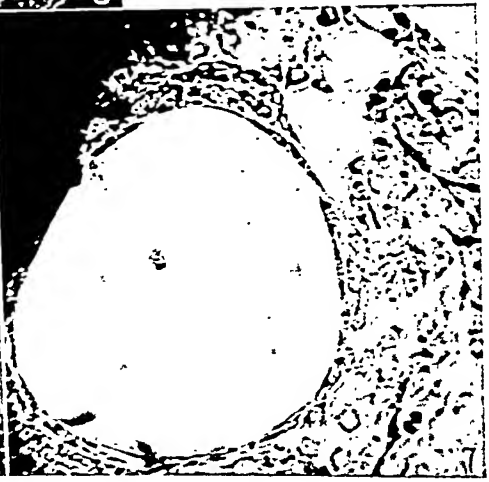

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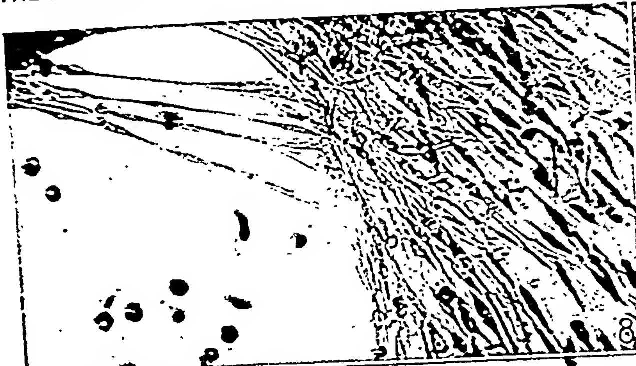
Photographed by Louis Schmidt

(Vaubel: Synovial cells in tissue cultures. I)



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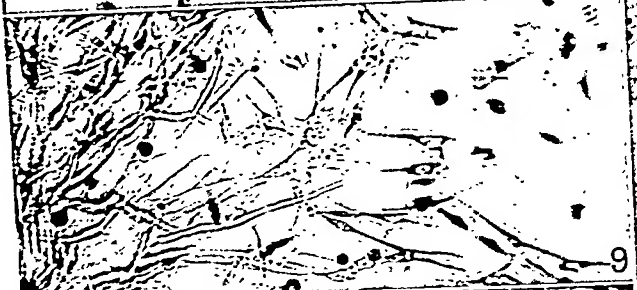

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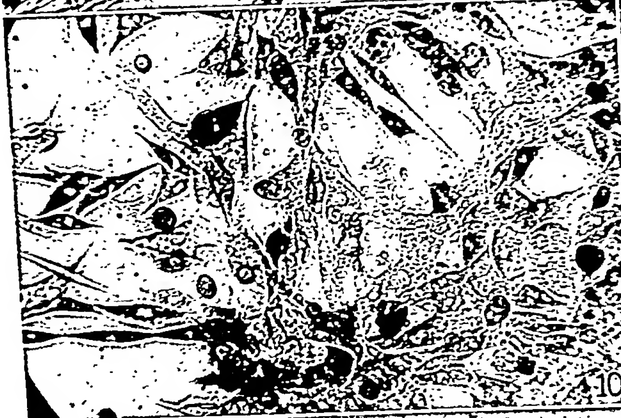

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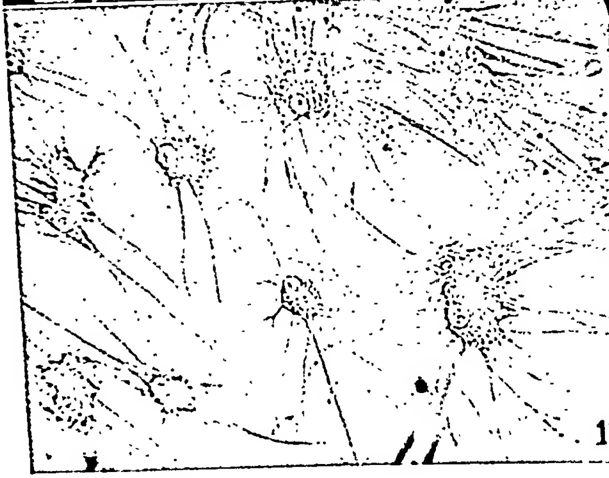

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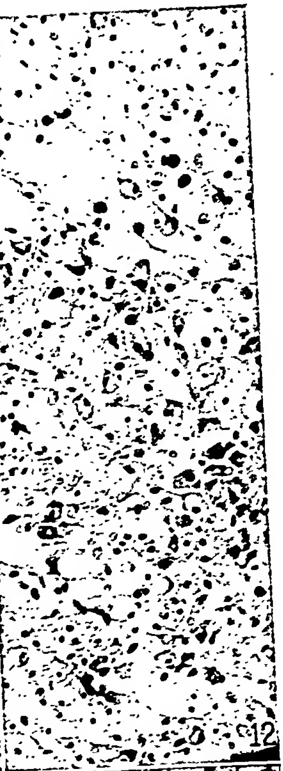

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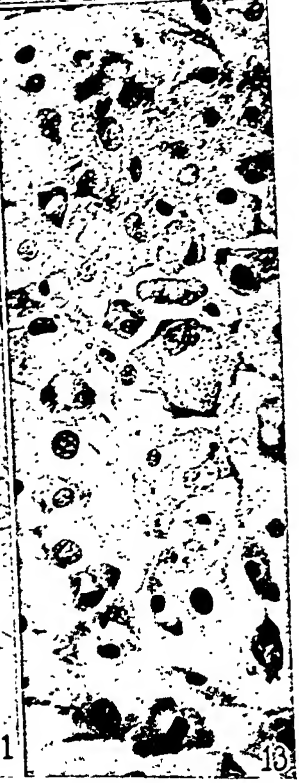

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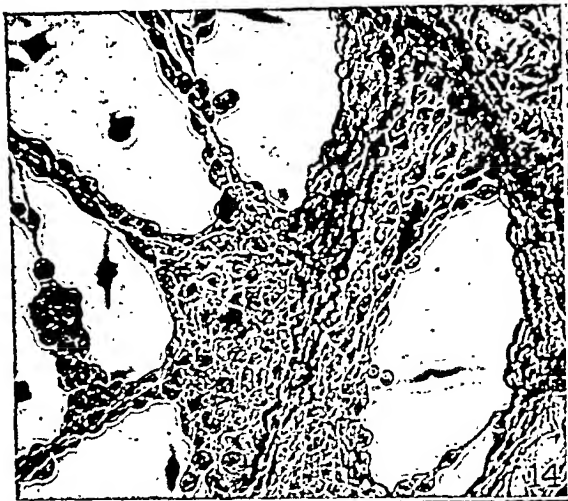
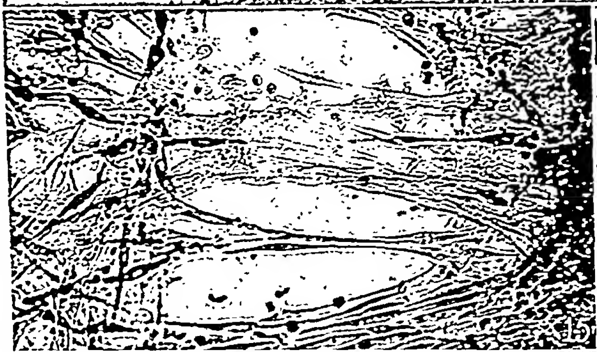
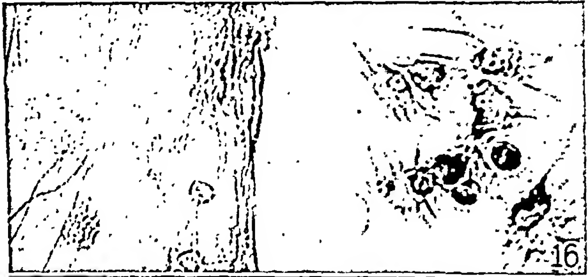
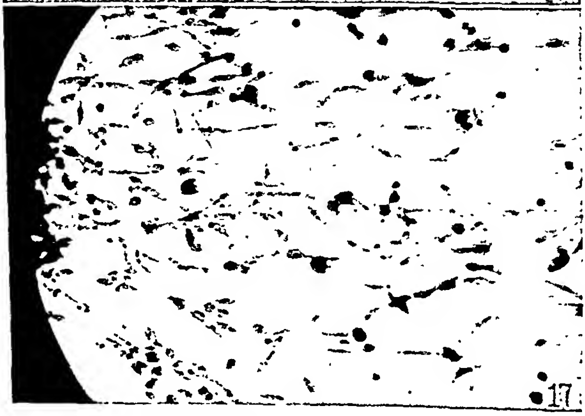
Photographed by Louis Schmidt

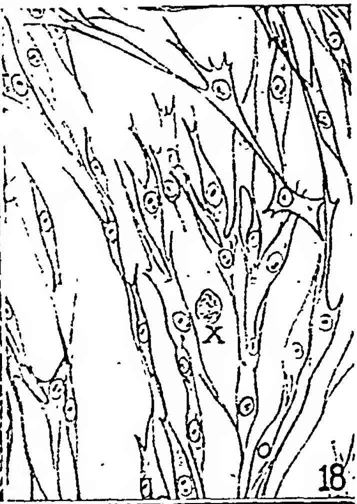
(Vaubel: Synovial cells in tissue cultures. I)

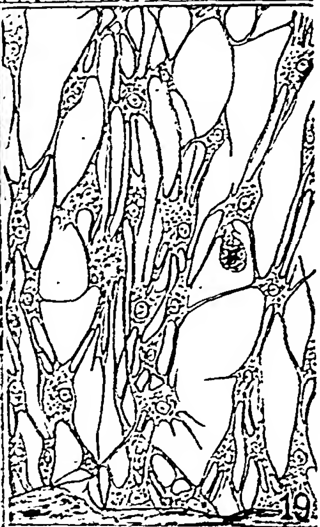


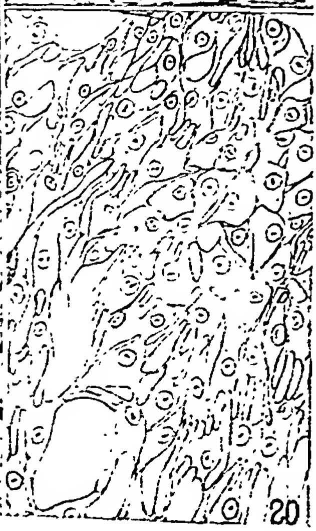












Photographed by Louis Schmidt

(Vaubel: Synovial cells in tissue cultures. I)

THE FORM AND FUNCTION OF SYNOVIAL CELLS IN TISSUE CULTURES

II. The Production of Mucin

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The object of this communication is to present evidence that mucin is actively produced by synovial cells. The demonstration of such a capacity by synovial cells in tissue cultures would be of importance because heretofore there has been no unanimity of opinion concerning the site and mode of formation of the synovial fluid, as is shown by the following brief review of the literature bearing on this subject.

In conformity with the idea that the synovial membrane was an epithelial structure the older authorities designated the joint fluid as a product of secretion in the narrower sense, a process observed only with genuine epithelial elements. The opinion was expressed by both Winslow (1) and Havers (53) that the synovial fatty tissue was a mucin-producing gland; but later Bichat (3) advanced opposing views, and he, together with other authors, saw in the joint fluid merely a transudate which percolated through the walls of the numerous blood vessels of the synovia (Bichat, Todd (54), Drechsel (55) and others); von Kölliker (2), on the other hand, thought that it was a fluid originating in the vessels but altered by some action on the part of the "synovial epithelium." Frerichs (56), who in 1846 first discovered mucin in the synovial fluid, and who considered the synovial membrane as anatomically composed of serosal cells, opposed the possibility of a secretory origin in the narrower meaning of the word, because these cells like those of all other serous membranes are capable of producing only an exudate. Thus the so called epithelial lining of the joint capsule was supposed to desquamate as a result of motion and then gradually to dissolve in the alkaline serum. This process was thought to be favored through the constant friction in the joints, a unique condition not existing in other serous membranes.

Frerichs' opinion that the mucinous nature of the synovial fluid was the result of cellular disintegration, and conditioned by the purely mechanical factor of motion, influenced many subsequent investigators, particularly those, such as Hammar (11), who regarded the lining of the joints as a simple fibrous connective tissue. Heuter (5), on the other hand, spoke of a specific action of the cells upon the nourishing fluid which transuded from the blood vessels, and found in the microscopic picture of the synovia no support for the idea that the cells are dissolved *en masse*. The secretion theory was, nevertheless, by no means dislodged. Thus, Soubbotine (12) stated that the synovial fluid was formed by goblet cells which he frequently encountered among the epithelial elements of the synovia; and Mayeda (13) described certain zones of the joint lining as secretory areas, characterized by glandular cells which he thought he could clearly differentiate both anatomically and functionally from those found in other places. Both Aschoff (14) and Kaufmann (15) speak of the synovial fluid as a secretion. Müller (57), basing his opinion on Lubosch's conceptions, expresses the contemporary viewpoint that the joint fluid must not be considered as entirely a secretion from the synovial membrane, but rather that it is chiefly a solution of cellular elements of the joint lining—a mucoid degeneration—that arises from a constant stream of fluid poured out from the articular surface. While some disciples of Frerichs still exist who consider the synovial fluid only as a substance arising from the mechanical destruction of the synovial cells, recently other voices have again been raised advancing arguments in favor of the secretion theory. These are based in part upon the results of histologic investigations (Mayeda (13), Kling (46)) and in part upon clinical and experimental researches. Noteworthy also is a theory advanced by Banchi (58), who regards the synovial fluid as originating from the articular cartilage rather than from the synovial membrane.

A study of the literature dealing with the embryologic phases of the subject, reviewed more extensively elsewhere (see footnote 1 in previous article), throws little light upon the question. Most of the authors subscribe to one or the other of the two viewpoints considered above. Only in Retterer's (59) writings does one encounter this interesting observation confirmed by all embryologic data, namely that the first trace of the synovial fluid takes its origin from a liquefaction of the hyaloplasm, and he concludes from this observation that a similar, peculiar liquefying condition must exist in postembryonic life.

In summary, then, it may be stated that there are two theories concerning the mode of formation of synovial fluid: the one tries to explain it as a secretion, the other considers it a product of cellular degeneration, in other words, a detritus dissolved in tissue juices. A satisfactory clearing up of these differences is not available; and because somewhat confused and unphysiologic contributions have led to theories not in accordance with contemporary knowledge, the whole subject requires revision.

A consideration of the foregoing opinions concerning the source of the synovial mucin leads to the following possibilities for experimental investigation. If the synovial fluid and especially its high content of mucin is a detritus arising from mucoid degeneration and cellular destruction then one would expect to find no mucin in a tissue culture composed of healthy synovial cells, but rather in a culture of degenerating synovial cells and possibly in one of fibroblasts grown from periarticular tissue. If, on the other hand, the mucin is a specific product of the synovial membrane one would expect to find a certain amount of it in a normally growing culture of synovial cells, and none in a degenerating growth or in a culture of fibroblasts. Finally, if the mucin arises as a result of degeneration of the cartilaginous portion of the joint then none would occur in a culture composed entirely of synovial cells.

EXPERIMENTAL

The same cultures, both of synovial membranes and of control tissues, used in the morphologic studies (60) were employed for the present study, for in this manner some of the forms and functions of the cells could be correlated.

The investigation of the soluble material originating in the tissue cultures was carried out in a simple manner. If the flasks contained no free fluid, either because no fluid phase had been supplied or because the plasma had absorbed the fluid previously added, the plasma coagulum was separated from the bottom of the flask thus allowing it to contract and squeeze out a certain amount of fluid; at times this process was hastened by centrifuging. The fluid so obtained was decanted or pipetted into small tubes and tested for mucin as described below. All control cultures were handled in the same manner; and in case the growth had progressed to the stage of complete degeneration similar procedures were employed for obtaining fluid. During a period of active growth when attempts were made to correlate possible mucin formation with a certain stage of growth a modification in the manner of collecting the fluid was introduced: the flasks were placed upright for half an hour when the expressed liquid which had collected at the dependent portion was removed with capillary pipettes; then that from each flask was tested separately or the product from a number of flasks was pooled and tested.

In order to eliminate the possibility of introducing mucin into the original culture media no embryonic extract was used. Furthermore, all fluids used in making and washing the cultures were tested for the presence of mucin with negative results. As already mentioned, all synovial membranes were washed half an hour in Tyrode's solution in order to remove adherent joint fluid before being explanted. Several times the explants before being placed in the plasma were washed with Tyrode's solution made slightly alkaline with sodium hydrate; and as an additional control, bits of synovial membrane were placed in similar alkalinized Tyrode's solution and incubated at 37°C. for 24 hours; the supernatant liquid tested with 2 per cent acetic acid never gave a positive reaction for mucin.

The test for mucin in a fluid under investigation was carried out as follows: to the clear solution, contained in small tubes, 2 per cent acetic acid was added drop by drop. In the presence of either mucin or nucleoprotein a precipitate is formed; but these precipitates have distinguishing characteristics: that formed by both appears to be composed of coarse threads, which, if the acetic acid is added carefully, gathers into a sack or tube-like membrane, as Kling recently demonstrated. If a glass rod is placed in the fluid the precipitate gathers around it in a pasty, elastic mass. If the precipitate is due to nucleoprotein it is soluble in an excess of acetic acid, while the presence of mucin is indicated by a failure of the precipitate to go into solution in an excess of the acid. The precipitated mucin is, however, readily soluble in alkaline media and can be repeatedly precipitated and redissolved by altering the reaction of the surrounding fluid.

Von Holst (61) states that the mucin of the synovia is free from phosphorus and yields a reducing substance after long heating with hydrochloric acid. Kling (46), in agreement with Salkowski (69), could demonstrate no noteworthy reduction with copper sulfate even after prolonged hydrolysis of the synovial mucin with dilute hydrochloric acid. Extensive chemical investigation of the mucinous material derived from tissue cultures was impossible because of the small amount of the substance available from such sources.

RESULTS

Before reporting in detail on the results with fluid obtained from synovial cultures it seems well to record the reaction obtained with that from controls. With cultures of healthy fibroblasts of different origin there occurred in no single case a precipitate of the coarse thready type; the solution usually remained clear following the addition of 2 per cent acetic acid or there occurred only a slight turbidity; such finely dispersed precipitates formed very slowly, often only after an interval of 24 hours. Fluid from the degenerated and dying cultures at times yielded a thread-like precipitate, small in amount, which redissolved upon the addition of an excess of acetic acid; usually, however, only a slight clouding was observed.

With fluid derived from serosal cell cultures from the pericardium, pleura, peritoneum and tunica vaginalis, similar results were obtained. In spite of varying cultural conditions no mucin-like substances were demonstrable, with the following exception: in the liquid from two flasks containing growths from explants of peritoneal serosa a precipitate was observed having the morphologic properties of mucin and not soluble in an excess of the acid. The amount of precipitate was too small for further chemical investigation. With numerous other cultures of peritoneal origin it was impossible to repeat this observation; nevertheless, it is of distinct interest, especially when considered in connection with the observation of von Holst, who demonstrated mucin-like substances in ascitic fluid.

In sharp distinction to the negative results noted above were the clearly positive findings with fluid obtained from cultures of synovial cells. By decanting and combining the liquid from several flasks a distinctly increased viscosity was observable; such fluid was like a thin glue and could be drawn into long threads; it was, indeed, very similar to a thin joint fluid. Upon the addition of 2 per cent acetic acid there was formed a coarse thready precipitate that quickly dropped to the bottom of the tube in the form of adherent clumps. This precipitate was insoluble in an excess of acid as well as in a neutral medium; it dissolved readily in slightly alkaline solutions, and again formed a precipitate upon the addition of an excess of acid to the alkaline solution; it was not coagulated by heat. At times a substance capable of reducing copper sulfate was obtained after prolonged heating of the precipitated material in dilute hydrochloric acid; but this result was inconstant, for the reaction was sometimes negative under experimental conditions apparently identical with those giving positive results.

The output of mucin was, naturally, small and not sufficient for extensive chemical studies; yet there seems little doubt of its identity with the mucin formed in joints. It did not give the reaction of nucleoprotein which dissolves in an excess of acid. Furthermore, in the fluids from degenerating synovial cultures there were found only traces of mucin or none, while the amount of thready precipitate of nucleoprotein soluble in an excess of acid was found to increase in such cultures.

The quantity of mucin precipitate varied, depending upon the proportion of producing cells and the number and extent of cultures in the flasks. It was especially marked with explants and with typically growing transplants, particularly in the older healthy cultures showing no cellular degeneration. The mucin could be demonstrated distinctly in fluid obtained from a sixth passage transplant, that is about 60 days after the original explantation. Its amount decreased with the increase of a fibroblast-like growth, and it disappeared with complete transformation of the synovial cells into typical fibroblasts, and no mucin appeared when such growths were allowed to degenerate. The transformation into fibroblasts with failure of mucin production seemed to occur more readily with transplants of synovia from young animals than with those from older rabbits. With the latter, as a rule, typical growths with mucin formation persisted through several passages.

When a flask was washed out with 1 cc. of Tyrode's solution on the day following explantation, no mucin, or at least only traces, could be detected in the washings; and the same was true of the supernatant liquid obtained by centrifuging the plasma coagulum in which explants 1 day old had grown. With progressing growth of typical synovial cells there was an increase in mucin production; after about 10 days, when the growths were divided and transplanted, production of mucin appeared to be at its maximum. If the culture was stimulated by the addition of a large amount of splenic extract then there was early a large yield of mucin; but under this stimulating environment the dedifferentiation into fibroblast-like growth took place more rapidly with a corresponding diminution in mucin production. By conditioning the nutritive state with small amounts of splenic extract the appearance of typically growing synovial cells was better maintained and the specific function of these cells seemed to be held at its optimum.

DISCUSSION

From a review of the results obtained in tissue cultures the conclusion seems warranted that cell types develop in the growths from synovial membranes which are clearly differentiated from other mesenchymal cells, especially from those of the subcutaneous or interstitial varieties. The peculiarity of a synovial growth rests in part on the polymorphism of the cells, the contour of which ranges from round and spindle to polygonal and epithelioid, and especially on their tendency to form membranes composed of syncytial elements; this agrees with histologic investigations (Hammar (11) and others). Furthermore, these cells are peculiar in producing both mucin and a proteolytic ferment and in forming in their cytoplasm large highly refractive granules, that stain deeply with neutral red. Because of these functions they are differentiated sharply from fibroblasts and brought into fairly close relationship with other cells of mesenchymal origin having special morphologic and physiologic features, namely osteo- and chondroblasts.

A comparison with the reports of other workers shows that these three types of cells appear to be related in several respects. Dolschansky's (62) descriptions and illustrations of growing osteoblasts and chondroblasts reveal a striking similarity to those given above for synovial cells. Fischer and Parker (18) confirmed Dolschansky's findings, and, in addition, caused both cell types under special cultural conditions to form an organized tissue with a hyalinized ground substance, that closely resembled bone or cartilage. The morphologic similarity of cultures of these three tissues is closely connected with their ontogenetic relationship: they arise from a common embryonic anlage.

The capacity of the synovial cells to dissolve the coagulated plasma under certain favorable circumstances may be regarded as a physiologic function of this tissue that may be likewise observed *in vivo.* For example, one finds references in the literature indicating that the synovial fluid is capable of dissolving bone when a bone pegging operation has been performed near a joint; and also when bits of bone have been broken off into the articular cavities. Coagulated blood in the synovial cavities becomes liquefied after a few days (Bier (63), Kaiser (64), Jaffé (65)). The poor healing of intraarticular fractures may also be cited in the same connection; and in order to explain this phenomenon Podkaminsky (66) investigated the lytic capacity of the fluid obtained from the joints of oxen and demonstrated *in vitro* a proteolytic ferment, a lipase and an amylase; he thought, therefore, that the occurrence of these substances accounted for the osteo- and chondrolytic power of the synovial fluid. The enzymes, especially those of proteolytic nature are doubtless active in supplying a medium suitable for the holding in solution of substances favorable to the peculiar function of the synovial membrane.

The demonstration of mucin in the cultures of synovial cells is important in indicating that the maintenance of the slimy property of synovial fluid is a peculiar function of these cells and not the result of their death and subsequent dissolution. The term "secretion" is avoided because mucin formation in joints cannot be compared strictly with the enzyme-forming function of epithelial glands, for which this term, in its narrower sense, should be reserved.

As an analogue to the formation of mucin by the synovial membrane one must look for the production of similar substances by certain other mesenchymal elements. This subject is discussed much more fully in another place (see footnote 1 in previous article), where the viewpoint is advanced that one may regard the synovial fluid as a ground substance of the synovial tissue analogous to the intercellular substance of the cartilage which has become solid by the imbibition of chondroitinsulfuric acid, or to that of the bones which have become impregnated with lime salts. This viewpoint is in perfect accord with the modern conception of mesenchymal tissue, as formulated by Hueck (67), Studnička (68) and others. The feature distinguishing this ground substance from all others is its persistent liquid mucinous state, a physical condition that renders it most valuable for the purposes of lubrication.

Finally a word concerning nomenclature may be introduced. We designate the individual elements of bone as osteoblasts, and those of cartilage as chondroblasts according as these cells elaborate a peculiar intercellular substance, bone or cartilage respectively. If, therefore, one regards the synovial fluid as a specific ground substance elaborated by synovial membrane it is only logical to employ the designation "synovioblast" to characterize a specific type of cell with this peculiar function.

SUMMARY

1. Synovial cultures are differentiated in tissue cultures from other tissues of mesenchymal origin by their type of growth and cell function.
2. In these respects they are more closely allied to chondroblasts and osteoblasts than to fibroblasts.
3. Synovial cells in tissue cultures develop marked globular cytoplasmic granulations that stain easily with neutral red and sometimes with toluidine blue; they show marked polymorphism with all transitions from round to spindle, polygonal and star shapes and eventually form an epithelial-like membrane, composed of cells with numerous syncytial bridges.

4. In cultures of typically growing synovial cells a mucin-like substance is elaborated. Typical growth and maximal mucin production is best maintained in media containing a minimum of growth-stimulating substances. Transformation of synovial cell growths into fibroblastic growth is accompanied by a loss of mucin production. Dying cells apparently do not produce mucin.

5. Amitotic cell division and the formation of macrophage-like cells were observed.

6. Marked tendency to liquefaction of the plasma about the growths was observed and attributed to the elaboration of a proteolytic ferment.

7. The specific designation "synovioblasts" is proposed for these cells.

The author wishes to express his thanks to Dr. Homer F. Swift for his encouragement during the course of this investigation, also for the translation of the articles from the author's German manuscript, and their preparation for publication.

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THE RACES THAT CONSTITUTE THE GROUP OF COMMON FIBROBLASTS

II. The Effect of Blood Serum

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Plates 7 to 10

(Received for publication, May 8, 1933)

The purpose of the experiments described in this article was to study the effect of serum on the morphology and rate of multiplication of strains of common connective tissue cells, or fibroblasts, and also to compare the action of serum with that of plasma.[1]

EXPERIMENTS AND RESULTS

When plasma is supplied to the cultures in a fluid state, it can be renewed at frequent intervals without disturbing the colonies. Coagulation is prevented by means of heparin. A comparison of the effects of serum and plasma was made possible, therefore, by the addition of this substance to both serum and plasma that had been prepared from a given sample of blood. Also, the effect of the heparin on the cells could be tested by comparing the action of plain serum with that of the same concentration of serum containing heparin.

Strains of fibroblasts were isolated from skeletal muscle (*M. tibialis anterior*) of 10 and 21 day old chick embryos and cultivated for seven passages (38 days) in flasks in a medium consisting of plain, adult chick plasma and chick embryonic tissue juice[2] diluted with Tyrode solution. On the final day of this treatment, by which time the cell population had been rendered uniform, each of a number of representative cultures from the two strains was divided into four equal and comparable parts, of which three were retained. From these, various series of experiments were made, each series comprising three sister cultures that were placed in

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[1] Parker, R. C., *J. Exp. Med.*, 1932, 55, 713.

[2] This was rendered cell-free by freezing for two periods of 15 minutes each at −50°C.

separate flasks in a medium consisting of 1 part of adult chick plasma diluted with 2 parts of Tyrode solution. Coagulation was allowed to proceed spontaneously and without the addition of tissue juice. After an incubation period of 24 hours, this solid phase was reinforced by a second application of plasma diluted as before with Tyrode solution. On the following day, all cultures were washed with Tyrode solution for 2 hours at 37°C., after which the three cultures comprising each set were treated with three different types of media: the first, with a mixture consisting of 1 part of chick serum diluted with 2 parts of Tyrode solution; the second, with a mixture of the same sample of serum diluted to the same extent with Tyrode solution but containing 1 part in 10,000 of a solution of heparin; the third, with plasma prepared from the same sample of blood and containing equal proportions of the same Tyrode and heparin solutions. As in the case of the Tyrode, these solutions were allowed to remain for 2 hours at 37°C. before being removed. Three times a week the cultures were again washed and treated with the same materials. When it became necessary to transfer them to fresh flasks, the solid medium was prepared as before and the daughter cultures continued to receive the same treatment as those from which they had been derived. At each transfer, it was aimed to have the three transplants of equal size.

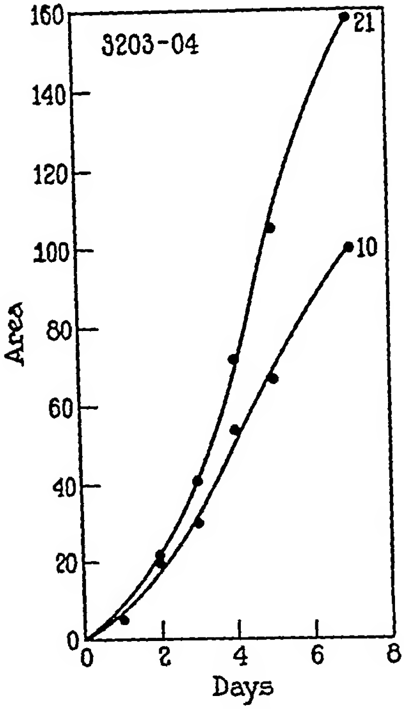
Whenever the culture flasks were opened for treatment, and before they were set away for incubation, they were filled with a gas mixture composed of 3 per cent CO_2, 21 per cent O_2, and 76 per cent N_2 (at atmospheric pressure). The effect of this was to adjust the hydrogen ion concentration of the various media to a pH of 7.6.

A second group of experiments was made from the strain that had been derived from the 21 day old embryo after this had been cultivated as "stock" (in a plasma-tissue juice medium) for ten passages (59–61 days).

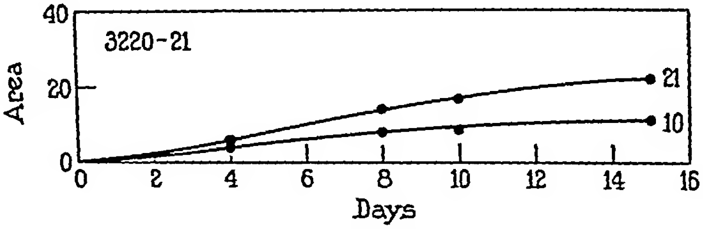
The 2-hour periods were chosen arbitrarily for the reason that they happened to fit best into the day's schedule. The length of time that a given quantity of plasma may be maintained in a fluid condition by the use of heparin depends entirely upon the concentration of the latter. These periods have been found adequate both for the removal of catabolic substances from the interstices of the solid medium and also for their subsequent replacement with fresh food materials. Were it feasible to increase the concentration of heparin, the nutrient fluids might well be left in place from one treatment until the following one 2 days later. The quantity of heparin this necessitates, however, is decidedly injurious to the cells.

The relative growth rates of the various cultures comprising each experiment were estimated from planimetric measurements of outline drawings made from time to time with the aid of the projectoscope. This apparatus, used as a camera, served to record their morphological appearance.

When cultivated under the same environmental conditions, the rate of cell multiplication displayed by the strain of muscle fibroblasts derived from the 21 day old embryo greatly exceeded that manifested by

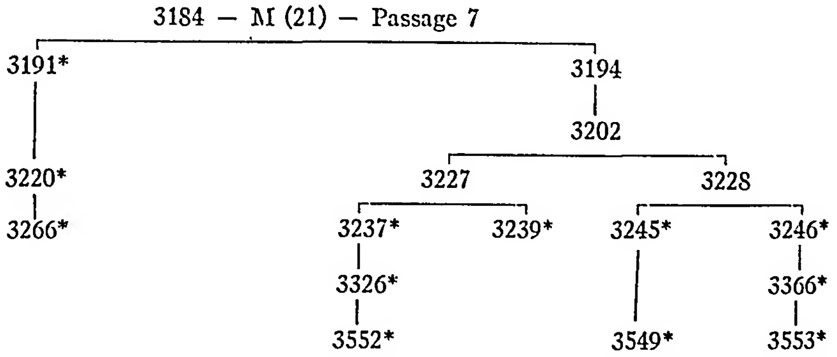


TEXT-FIG. 1. Curves showing the rate of growth of two strains of fibroblasts isolated simultaneously from the *M. tibialis anterior* of a 10 and a 21 day old chick embryo, respectively, and cultivated for 52 days (nine passages) in chick plasma and chick embryonic tissue juice.



TEXT-FIG. 2. Curves showing the rate of growth of two strains of fibroblasts isolated simultaneously from the *M. tibialis anterior* of a 10 and a 21 day old chick embryo, respectively, and cultivated in heparinized serum. From isolation until the present period, the two strains were cultivated for 38 days (seven passages) in embryonic tissue juice, and subsequently for 14 days (one passage) in heparinized serum.

the strain obtained from the younger embryo. This was true not only when the two strains were cultivated in a highly nutritive medium (Text-fig. 1), but also when the food content of the medium was of a lower level, as, for example, that represented by serum or plasma (Text-fig. 2).



Text-Fig. 3. Schematic representation of relationships existing between cultures of the various experiments for which data are supplied in Table II.

* Three sister cultures treated with serum, heparinized serum, and heparinized plasma, respectively.

TABLE I

Action of Serum, Heparinized Serum, and Heparinized Plasma on the Rate of Growth of Fibroblasts from the M. tibialis anterior of a 10 Day Chick Embryo

M(10) experiments	Passage No.	Duration of treatment	Area of outgrowth attained at end of each passage		
			Serum	Serum (heparinized)	Plasma (heparinized)
		days			
3183*	7				
↓ 3192	8	14	19	23	12
↓ 3221	9	64	71	33	23
↓ 3596	10	22	29	27	

* Final passage in embryonic tissue juice.

The organization of the serum and plasma experiments selected for presentation, and the results obtained, are presented in Text-fig. 3, Tables I and II, and Figs. 1–17. Six series of experiments are included, one utilizing the strain derived from the 10 day old embryo (Table I, Figs. 4–15), whereas the remaining five include the strain derived from the older embryo (Text-fig. 3, Table II, Figs. 1–3 and 16 and 17).

Each series was started from a culture belonging to a set that had been cultivated up to that point in the routine stock medium of plasma and embryonic tissue juice (compare Text-fig. 3 with Table II).

TABLE II

Action of Serum, Heparinized Serum, and Heparinized Plasma on the Rate of Growth of Fibroblasts from the M. tibialis anterior of a 21 Day Chick Embryo

M(21) experiments	Passage No.	Duration of treatment	Area of outgrowth attained at end of each passage		
			Serum	Serum (heparinized)	Plasma (heparinized)
		days			
3184*	7				
↓ 3191	8	14	27	34	18
↓ 3220	9	17	21	22	9
↓ 3266	10	40	63	45	
3227*	10				
↓ 3237	11	26	42	42	
↓ 3326	12	24	60	40	
↓ 3552	13	24	47	46	
3227*	10				
↓ 3239	11	25	72	43	40
3228*	10				
↓ 3245	11	48	116	88	31
↓ 3549	12	24	74	47	
3228*	10				
↓ 3246	11	28	66	45	
↓ 3366	12	20	43	46	
↓ 3553	13	24	46	46	

* Final passage in embryonic tissue juice.

The Effect of Serum, Heparinized Serum, and Heparinized Plasma on the Rate of Cell Multiplication

An examination of the data presented in Tables I and II will reveal that, with one exception (Experiment 3191), the fibroblasts cultivated on serum alone attained an area of outgrowth either greater than or equal to that obtained by the use of heparinized serum. In the case of this one exception, however, it will be noted that, for the two subsequent passages during which transplants of these cultures were subjected to the same type of treatment as had been afforded the parent cultures, the results were comparable with those of the other experiments. It will also be seen that those cultures treated with heparinized plasma showed less growth than those that had received either the heparinized serum or the serum treatment.

Previous experiments,[1] together with those to be reported here, have demonstrated that fibroblasts can be cultivated in heparinized plasma for at least 13 months. Since no advantage was to be gained by carrying them long in these particular experiments, the plasma cultures were early discarded. Those treated with heparinized serum were next discarded, but not until they had been successfully cultivated in this medium for 100 days. The cultures treated with plain serum were retained. At the time of writing, they have continued to multiply in this medium for 206 days.

The Effect of Serum, Heparinized Serum, and Heparinized Plasma on the Morphology of the Cells

Whereas the fibroblasts derived from the 21 day old embryo multiplied more rapidly than those obtained from the 10 day old embryo, the cells of both races showed much the same morphological appearance after having been cultivated in serum (Figs. 1 and 4), heparinized serum (Figs. 2 and 5), and heparinized plasma (Figs. 3 and 6), respectively, for the same length of time. As might be expected, the cells possessing the higher residual growth energy[3] showed, in each case, slightly less granulation than those endowed with a lesser capacity for multiplication in a given medium.

The cells cultivated in serum contained fewer granulations and revealed less evidence of degeneration than those cultivated in the same sample of serum containing heparin. Also, the cells treated with heparinized serum were at all times and in all cases in much better condition than those whose nourishment consisted of heparinized plasma. As will be emphasized later, these differences were particularly noticeable during the first days of cultivation in the three types of media. Later,

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[3] Carrel, A., *J. Exp. Med.*, 1923, 38, 521.

interesting changes occurred. In the case of sister cultures treated regularly with serum and heparinized serum, respectively, the pronounced morphological differences that had previously characterized the two types of treatment gradually disappeared. In both cases, the new cells were of a more healthy appearance, contained fewer inclusions, and did not tend to degenerate as rapidly as those that had preceded them. Thus, to draw examples from a single series of experiments, a culture that had been treated with serum for 27 days (14 days in the previous passage; 13 days in the current passage) was, at the end of that time, in a much healthier state than a sister culture that had received heparinized serum for the same period (Figs. 4 and 5). After 11 days, the difference between the two was far less (Figs. 7 and 8). When the treatment had been continued for 56 days, it was almost impossible to distinguish them morphologically (Figs. 10 and 11). On the 64th day, because of an increasing opacity of the medium, it became necessary to transfer the two cultures to fresh flasks. After the experiment had been continued for 14 days in the new passage, or 92 days from the time the various treatments were instituted, the two cultures were indistinguishable both as to morphology and their rate of growth (Figs. 14 and 15). Whereas the serum culture had attained an area of outgrowth that was over twice the size of the other in the previous passage, the two cultures were of almost equal area in this final passage. This was largely due to a definite increase in the activity of the culture that had been fed with heparinized serum and, to a lesser extent, a slight retardation on the part of the serum culture.

The change that occurred in the third culture of this series, namely that treated with heparinized plasma, was even more marked. After 27 days, the marginal cells were quite lifeless (Fig. 6). Inasmuch, however, as such cultures are not necessarily dead throughout,[1] the treatment was continued. On the 42nd day, the same disintegrated structures were observed on the same points of the margin (Fig. 9), memorials, as it were, to cells that had long since succumbed. Yet the culture continued to be fed three times a week along with the others. On or about the 50th day, new cells were seen to appear among the dead skeletons. These continued to migrate out from the interior of the culture and to multiply to such an extent as eventually to obscure from view the remnants of those cells that had preceded them weeks before (Fig. 12). With the passage of time, the new cell population became more and more interesting and gradually included a great diversity of forms (Fig. 13). With the aid of the camera lucida, many of the cells were observed to pinch off one or more small cell-like bodies. In many respects, this latter phenomenon resembled one that had been observed in previous experiments in which fibroblasts gave rise to true macrophages.[1] In the present case, however, the structures that were budded off bore no likeness to macrophages and remained active for but a short time.

A somewhat similar change was found to occur in various cultures treated with heparinized serum. Here, the outermost marginal cells gradually degenerated, forming a heavily granulated ring that completely encircled the culture (Fig. 16). After a time, living cells migrated out from within, as in the case of the culture described above. There was, however, no fragmentation. As these cells continued to multiply and to migrate outward, the narrow ring of degenerated cells, always visible, was left farther and farther behind (Fig. 17).

The Effect of Heparinized Plasma on the Growth and Multiplication of Heart Fibroblasts Belonging to Carrel's 21 Year Old Strain

Since fibroblasts derived from the musculature of the heart possess a residual growth energy[3] that is almost as low as that of any that have been isolated from the chick embryo, it was of interest to test their reaction to heparinized plasma under the conditions of the present experiments. The coagulum employed at this time differed from that of the preceding experiments in that a trace of embryonic tissue juice[2] was added to the original plasma and Tyrode mixture in order to promote rapid coagulation. This was done for the reason that these experiments were conducted with others involving the cultivation of a pure strain of macrophages, and according to identical procedures. In all other respects, the treatment was the same as that already described.

The fibroblasts were successfully cultivated in this manner for 96 days (Fig. 18). At the end of that time, the experiment was terminated by accident. Nevertheless, the fact that it was found possible to cultivate heart fibroblasts for so long a period indicated that these cells, under appropriate conditions, can and do utilize serum and plasma proteins for their growth and multiplication. It is a well known fact that a relatively high concentration of embryonic tissue juice is necessary for the continued well-being of all strains of fibroblasts derived from the embryonic heart when cultivated in the absence of serum and plasma proteins. Hence, it may be assumed that, in this instance, their continued multiplication for 96 days was not due to the infinitesimal amount of tissue juice used in the preparation of the solid medium. Even if the concentration used had been sufficient for cell growth and multiplication, it would have been washed out at the beginning of each passage and prior to the first treatment with heparinized plasma.

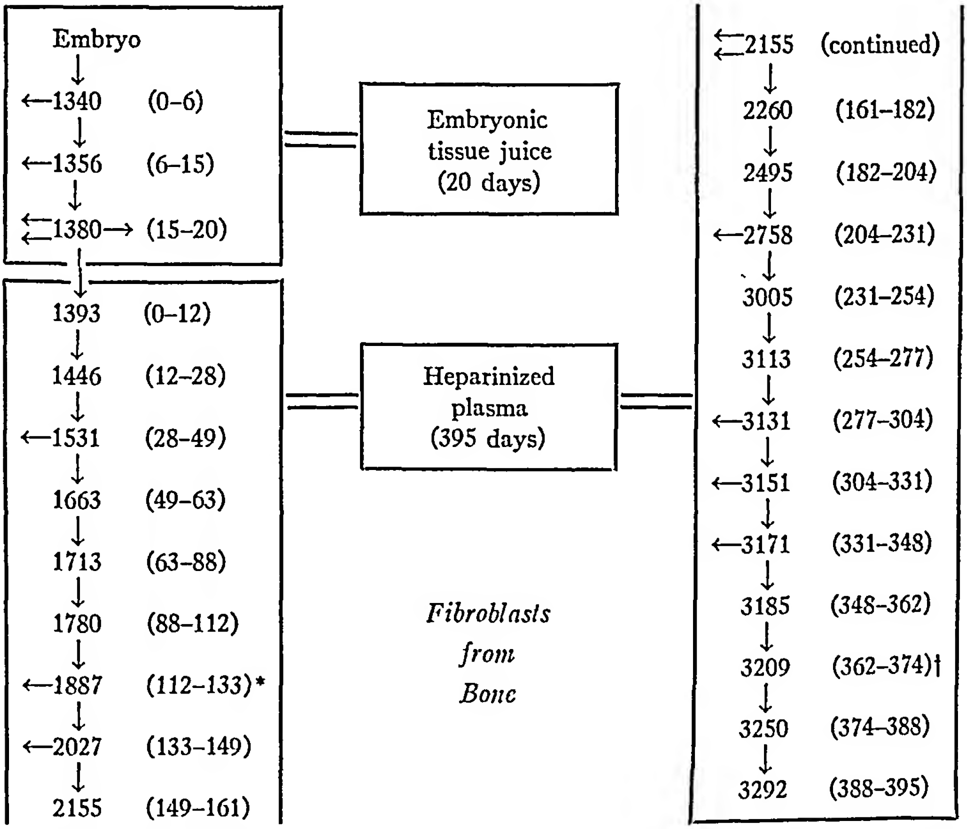
A Strain of Fibroblasts from Bone Cultivated in Heparinized Plasma for 395 Days

Fibroblasts derived from the periosteum of bone removed from the skull of a 13 day old chick embryo were cultivated for three passages (20 days) in a medium containing chick plasma and chick embryonic tissue juice diluted with Tyrode solution. On the final day of this treatment, by which time the cell population had been rendered uniform, several cultures from the series were placed in flasks in a medium consisting of 1 part of chick plasma diluted with 2 parts of Tyrode solution. No embryonic tissue juice was added. A day later, the coagulum was reinforced by a second layer of plasma diluted as before with Tyrode solution. On the following day, and three times a week thereafter, the cultures were washed for 2 hours with Tyrode solution, after which they were treated for a similar period with a mixture consisting of 1 part of chick plasma diluted with 2 parts of Tyrode solution and containing 1 part in 10,000 of a solution of heparin. Whenever it became necessary to transplant the cultures to fresh flasks, the solid medium was prepared as before and the same manner of treatment was resumed.

Under these conditions, the strain was cultivated for 395 days (Text-figs. 4 and 5, Fig. 19). At the end of that time, it was again treated with embryonic juice to determine whether or not the cells would revert to the form they had assumed over a year before and prior to their cultivation in the total absence of tissue juice. This they did. Not only were they stimulated immediately to more rapid division, but the cell population became more uniform in appearance.

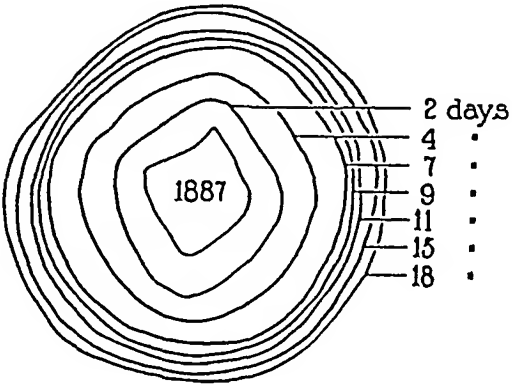
The Effect of Serum and Plasma from a 5 Year Old Hen on Fibroblasts from Skeletal Muscle

A strain of fibroblasts derived from skeletal muscle of an 11 day old embryo was cultivated for twelve passages in a medium consisting of



TEXT-FIG. 4. Schematic history of a strain of fibroblasts isolated from the periosteum of a 13 day old embryo and cultivated for 20 days in embryonic tissue juice and subsequently for 395 days in heparinized plasma. The arrows indicate the number of subcultures made at each transfer.

* See Text-fig. 5. † See Fig. 19.



TEXT-FIG. 5. Diagrammatic representation of the increase in area of a culture of fibroblasts isolated from the periosteum of a 13 day old embryo, and belonging to a series that had been cultivated in heparinized plasma for 112 days. (Compare Text-fig. 4.)

chick plasma diluted with Tyrode solution and chick embryonic tissue juice. At the end of this period, each of a series of representative cultures from the strain was divided into four equal parts. The present communication is concerned with but two of the four cultures that were prepared from each set. One of these was treated regularly with serum that had been obtained from the blood of a 5 year old hen and diluted with Tyrode solution; the other, with a mixture of heparinized plasma prepared from the same sample of blood and diluted, as before, with Tyrode.

This experiment has been included to show the appearance of the cell colonies during the first period of administration of serum and plasma and also to indicate the extent to which the action of the two substances may differ. After 2 weeks, the difference in the effect produced by them was enormous (Figs. 20 and 21). The cells cultivated in serum were in much the better condition. Those treated with heparinized plasma became so distended with inclusions as almost to lose their identity.

The Effect of Serum on the Duration of Life of Fibroblasts

Fibroblasts derived from skeletal muscle have been cultivated in serum and Tyrode solution, according to procedures herein described, for a total period of 206 days. One of these cultures (Experiment 3596, Table I), although treated regularly with fresh serum, has been retained in the same flask without subculturing for the last 128 days of this period. The colony is still active and comprised of cells that are in excellent condition. With the passage of time, its cell population has become more and more interesting. In the beginning, the marginal cells were uniform in appearance, conforming, in this respect, to the conventional fibroblastic type. This may be seen in the photographic record that was made of the culture after it had been carried for 14 days in the passage under present consideration (Fig. 14). By the time the serum treatment had been continued for 60 days, however, the cell population had become exceedingly heterogeneous. Side by side with degenerating cells, heavily distended with large accumulations of granules, were to be seen cells that were remarkably clear and free from gross cytoplasmic inclusions. Large groups of these cells increased in size, became stellate, and developed numerous radial processes (Figs. 24–27). The latter became entangled, forming bizarre, lace-like patterns (Fig. 24). Unlike cells cultivated under conditions conducive to rapid multiplication (Fig. 22), there was in this case no discernible polarity of the cells (Fig. 23). If the cells actually possessed an organic axis, its direction was vertical to their surface.

DISCUSSION

The present experiments have shown that fibroblasts, in the beginning, multiply more abundantly in plain serum than in heparinized serum, and also more abundantly in heparinized serum than in heparinized plasma. Any of these combinations, however, affords the materials necessary for cell growth. Thus, fibroblasts from bone have continued to multiply for 13 months in a medium consisting solely of heparinized plasma and Tyrode solution.

The readiness with which fibroblasts are able to utilize serum and plasma proteins depends entirely upon the nature of the strain. Cell races endowed with a high residual growth energy[3] are better able to multiply at the expense of these substances than are those whose growth potencies are of a lower order. Yet fibroblasts from the heart have been successfully cultivated in heparinized plasma for 96 days. These cells possess a residual energy which is almost as low as that of any that have been isolated from the embryo chick.

It has been shown that the first effect of serum, heparinized serum, and heparinized plasma on fibroblasts is invariably injurious, the degree of injury differing according to the race and medium employed. This is obviously due to the action of the inhibiting substances that have been shown by Carrel and Ebeling to be present in the plasma of adult and old animals.[4] Before being placed in these various media, the cells had been cultivated in embryonic tissue juice. During this period of plenty, they accumulated reserves that served to diminish the shock of the less favorable environment to which they were transferred. They would soon have succumbed had it not been possible for them to derive nourishment from serum and plasma. In certain cultures treated with heparinized plasma, it seemed as if this were to be the case. Although they were washed and fed at frequent intervals, the cells comprising their marginal areas gradually degenerated and finally died. After a time, however, and even in instances where the

[4] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, **34**, 317; 1923, **37**, 653, 759.

treatment was continued for as long as 25 to 30 days after the cultures had ceased to grow, new cells began to migrate out from the interior of the culture and to form a broad band of new growth out beyond the dead margin. In other cases, particularly in cultures treated with serum, the degeneration process advanced less rapidly and was always less pronounced. Yet here, as well, the cells underwent a gradual but very marked improvement. Successive generations of new cells were in better condition than the preceding ones and remained so for a longer time.

Carrel and Ebeling[5] have already demonstrated that the growth-promoting and growth-inhibiting qualities of serum and plasma differ with the age of the animal from which they are obtained. This has been seen again in the present experiments. The effect of serum and plasma prepared from the blood of a 5 year old hen is far more pronounced than the effect of the same materials derived from the blood of a younger animal.

Zakrzewski[6] has recently reported experiments in which he cultivated mesenchyme cells from mouse, rat, and chick embryos in plain blood serum for a maximum period of 29 days, and in heparinized serum and heparinized plasma for longer intervals. He concludes from his results that heparinized serum is a better source of nutriment for the cells than serum to which heparin has not been added. Serum, he states, contains both food substances and growth-stimulating substances, the latter in the form of prothrombin. Heparin, as antiprothrombin, inhibits growth by offsetting the action of the prothrombin. This, according to him, makes for a state of equilibrium and promotes functional differentiation. When heparin is absent, however, the cells are stimulated by the prothrombin to a rate of division that is too great for the amount of food present in the serum. The result is cell death.

These findings of Zakrzewski[6] have not been confirmed. Fibroblasts have been cultivated in plain serum and Tyrode solution for over 6 months. If heparin is a beneficial adjunct to the cultivation of cells in serum, they are able, eventually, to do without it. If, on the other hand, its effect is merely a toxic one, the cells are equally able to overcome this. It has already been noted that the growth of fibroblasts in heparinized serum is less at first than in plain serum. Finally, how-

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[5] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, 34, 599; 1922, 35, 17; 1923, 38, 419.

[6] Zakrzewski, Z., *Arch. exp. Zellforsch.*, 1932, 13, 152.

ever, cultures that have been treated for long periods of time in the two substances become indistinguishable both with respect to their general appearance and their rate of proliferation. The experiments of Zakrzewski were terminated before the cells had become fully adjusted to the serum medium.

It is now possible to understand and to evaluate the negative results obtained by Carrel and Ebeling[4] in their early attempts to cultivate fibroblasts indefinitely in serum. The majority of these experiments were made in hanging drops. In such a system, the volume of medium was so small in comparison with the mass of tissue it contained that the cell colonies had to be transferred every few days in order that a sublethal concentration of catabolites might be maintained. Under these conditons, the inhibiting qualities of serum were greatly exaggerated. Serum retarded the rate of cell multiplication. This, coupled with the necessity of sacrificing a large part of the marginal outgrowth at each transfer, meant that the mass of the colony was rapidly diminished by the very process of endeavoring to keep it alive.

Nevertheless, the older techniques fully satisfied the demands that were made upon them. As has just been noted, they served, very adequately, to demonstrate those qualities of serum that are responsible for the inhibition of growth.[4] Whereas, however, the hanging drop system offered one particular set of experimental conditions, the newer flask procedure of Carrel[7] provided still others. The present experiments, for example, have shown it to be unnecessary to maintain fibroblasts in a state of rapid division in order to keep them alive *in vitro.* As soon as the cells are permitted to remain undisturbed in some system that makes for slow multiplication, and at the same time allows a frequent interchange of materials, they are able to overcome the growth-inhibiting effects of serum and plasma and to utilize to better advantage the growth-promoting substances that are present.

Fibroblasts, then, proliferate rapidly when provided with proteins of the embryo, less rapidly in media containing serum and plasma proteins. In a given medium, each cell type shows a certain specific growth energy. This growth energy remains constant as long as the

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[7] Carrel, A., *J. Exp. Med.*, 1923, 38, 407.

medium permits the constant production of like individuals. In a medium containing an abundance of growth-activating substances as, for example, when embryonic tissue juice is present, the cells either reproduce themselves at frequent intervals, or die. After a certain number of days, even when the medium is replenished regularly, growth will cease and degeneration will set in. At any given moment, therefore, both the individual colonies and their component cells will have the same general appearance. In serum, however, multiplication is far more infrequent than before and cell death becomes an unusual occurrence. The majority of the cells lose their typical fibroblastic form. Furthermore, they produce colonies that differ from one another, both with respect to their general appearance and the nature of their component elements.

SUMMARY AND CONCLUSIONS

1. Under appropriate conditions, fibroblasts are able to multiply in serum, at a slow rate, for very long periods.
2. The rate of multiplication of fibroblasts in a given sample of serum depends entirely upon the nature of the strain. Cell races endowed with a high residual growth energy multiply more rapidly in serum than those whose growth potencies are of a lower order.
3. Fibroblasts, in the beginning, multiply more abundantly in plain serum than in heparinized serum, and also more abundantly in heparinized serum than in herparinized plasma. Later, these differences become less pronounced.
4. The first effect of serum on fibroblasts is invariably injurious, the degree of injury differing according to the nature of the cell strain and the age of the animal from which the serum is derived. With the passage of time, however, the colonies undergo gradual improvement, both in the appearance of the component cells and in their rate of proliferation.
5. In media containing embryonic tissue juice, or other growth-activating substances, fibroblasts form colonies that are isomorphic and composed of isomorphic cells. In serum, fibroblasts form colonies of heteromorphic appearance. Each colony becomes composed of cells that differ from one another, to a more or less marked degree.

EXPLANATION OF PLATES

PLATE 7

FIG. 1. Culture 3220-1. Fibroblasts from muscle of 21 day old embryo cultivated for 27 days in serum. × 110.

FIG. 2. Culture 3220-2. Sister culture after 27 days in heparinized serum. × 110.

FIG. 3. Culture 3220-3. Sister culture after 27 days in heparinized plasma. × 110.

FIG. 4. Culture 3221-1. Fibroblasts from muscle of 10 day old embryo cultivated for 27 days in serum. × 110.

FIG. 5. Culture 3221-2. Sister culture after 27 days in heparinized serum. × 110.

FIG. 6. Culture 3221-3. Sister culture after 27 days in heparinized plasma. × 110.

FIG. 7. Culture 3221-1. Same as Fig. 4 after 38 days in serum. × 110.

FIG. 8. Culture 3221-2. Same as Fig. 5 after 38 days in heparinized serum. × 110.

FIG. 9. Culture 3221-3. Same as Fig. 6 after 42 days in heparinized plasma. × 110.

PLATE 8

FIG. 10. Culture 3221-1. Same as Figs. 4 and 7 after 56 days in serum. × 110.

FIG. 11. Culture 3221-2. Same as Figs. 5 and 8 after 56 days in heparinized serum. × 110.

FIG. 12. Culture 3221-3. Same as Figs. 6 and 9 after 56 days in heparinized plasma. × 110.

FIG. 13. Culture 3221-3. Same as Figs. 6, 9, and 12 after 59 days in heparinized plasma. × 110.

FIG. 14. Culture 3596-1 (from No. 3221-1). Same strain shown in Figs. 4, 7, and 10 after 92 days in serum. × 110.

FIG. 15. Culture 3596-2 (from No. 3221-2). Same strain shown in Figs. 5, 8, and 11 after 92 days in heparinized serum. × 110.

PLATE 9

FIG. 16. Culture 3239-2. Fibroblasts from muscle of 21 day old embryo cultivated for 20 days in heparinized serum. × 110.

FIG. 17. Culture 3245-2. Sister culture after 34 days in heparinized serum. × 9.

FIG. 18. Culture 13517 D1. Heart fibroblasts from 20 year old strain cultivated for 96 days in heparinized plasma. × 220.

FIG. 19. Culture 3209. Fibroblasts from periosteum of bone from 13 day old embryo cultivated for 371 days in heparinized plasma. (Compare Text-fig. 4.) × 110.

FIG. 20. Culture 1271-1. Fibroblasts from muscle of 11 day old embryo cultivated for 14 days in serum. × 110.

FIG. 21. Culture 1271-2. Sister culture after 14 days in heparinized plasma. × 110.

FIG. 22. Culture 3964-6. Fibroblasts cultivated in embryonic tissue juice; inner zone of outgrowth. (Compare Fig. 23.) × 220.

FIG. 23. Culture 3596-1. Fibroblasts cultivated in serum; colony regularly treated without subculturing for 60 days; inner zone of outgrowth. (Compare Fig. 22.) × 220.

PLATE 10

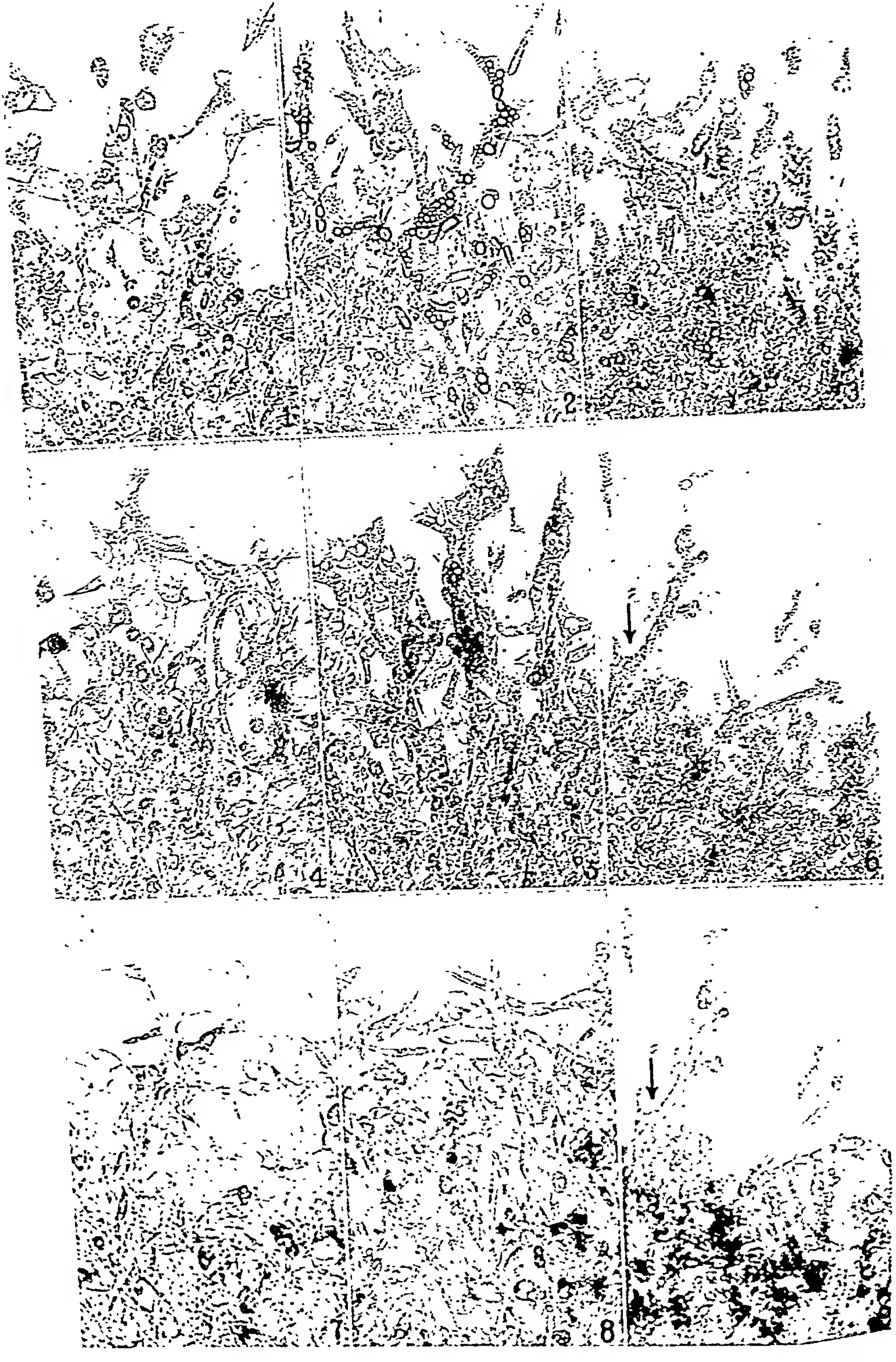
FIG. 24. Culture 3596–1. Marginal cells from culture shown in Fig. 14, 46 days later. This colony has not been subcultured for 60 days. × 220.

FIG. 25. Culture 3596–1. Marginal cell from culture shown in Figs. 23 and 24. × 220.

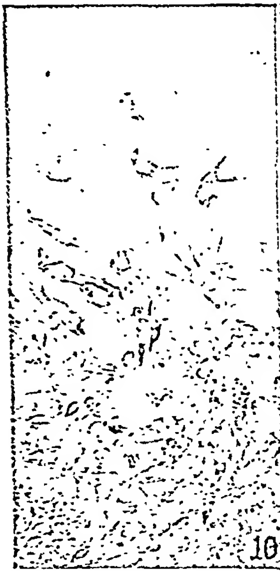
FIG. 26. Culture 3596–1. Marginal cells from culture shown in Figs. 23–25. × 220.

FIG. 27. Culture 3596–1. Marginal cell from culture shown in Figs. 23–26, 66 days later. This colony has not been subcultured for 126 days. (At this point, the strain has been cultivated for a total period of 204 days in serum.) × 220.

PLATE 7



(Parker: Races constituting common fibroblasts. II)



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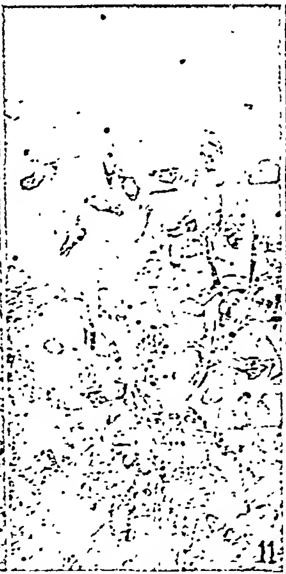

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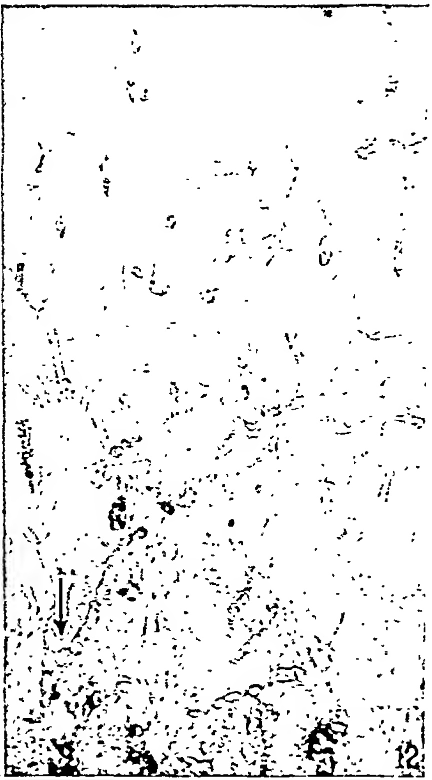

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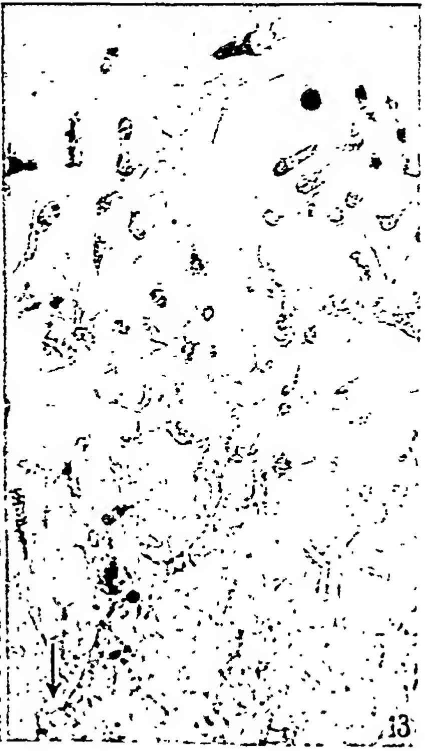

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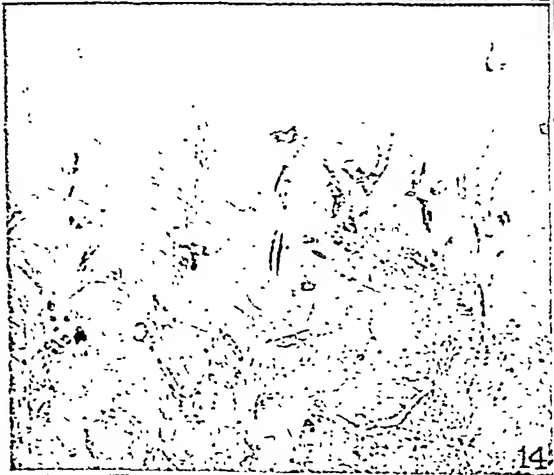

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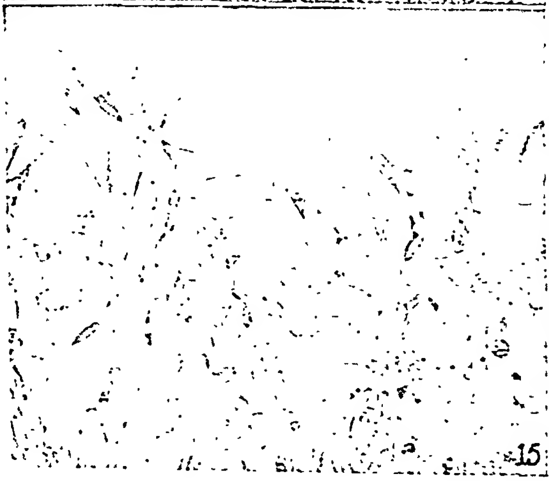

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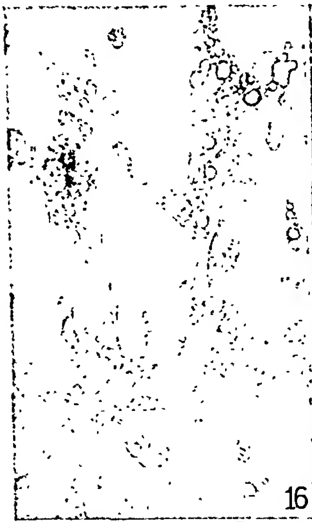
(Parker: Races constituting common fibroblasts. II)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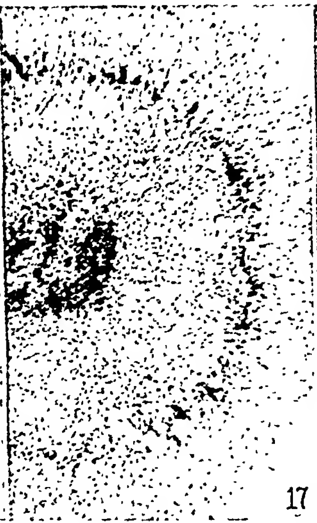
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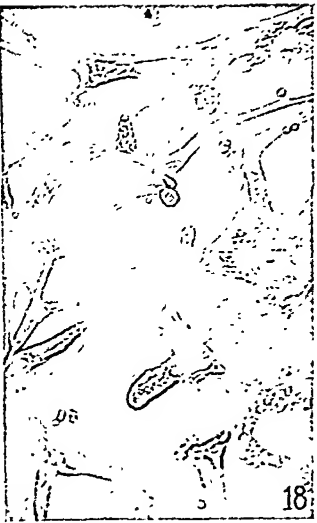
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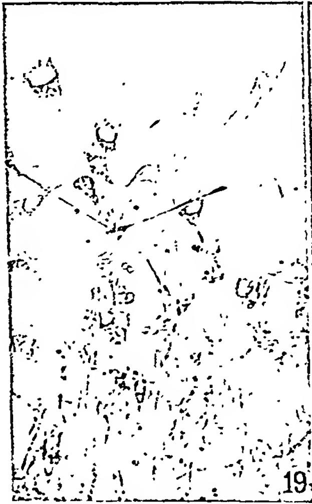
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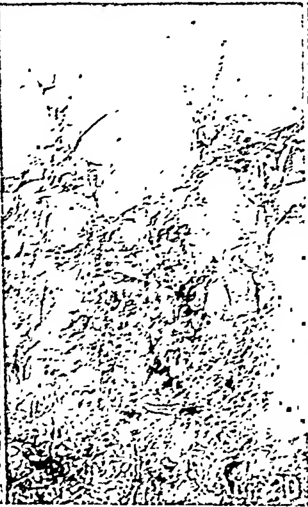
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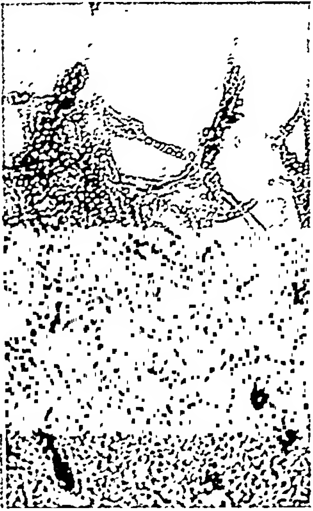

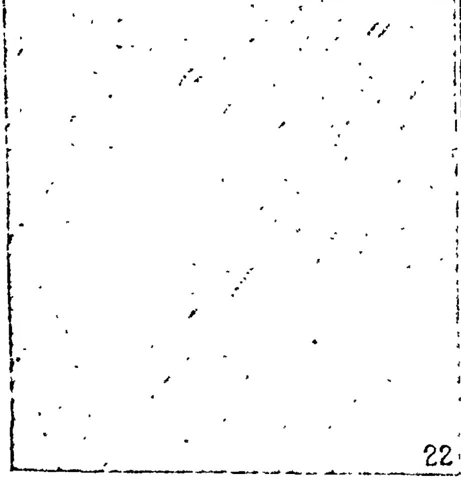
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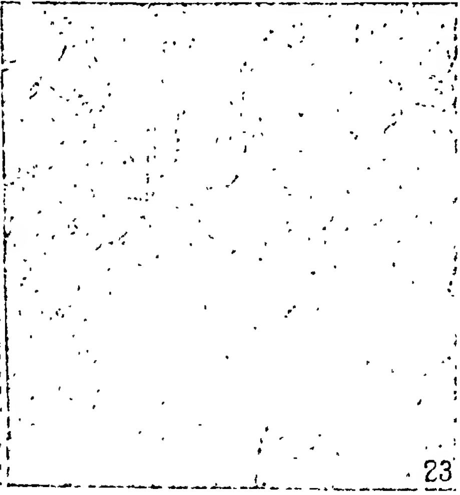
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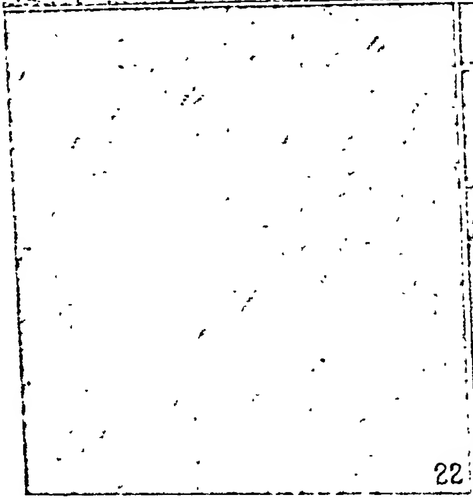

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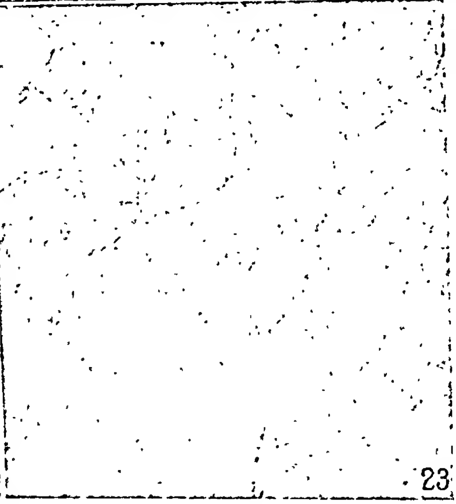

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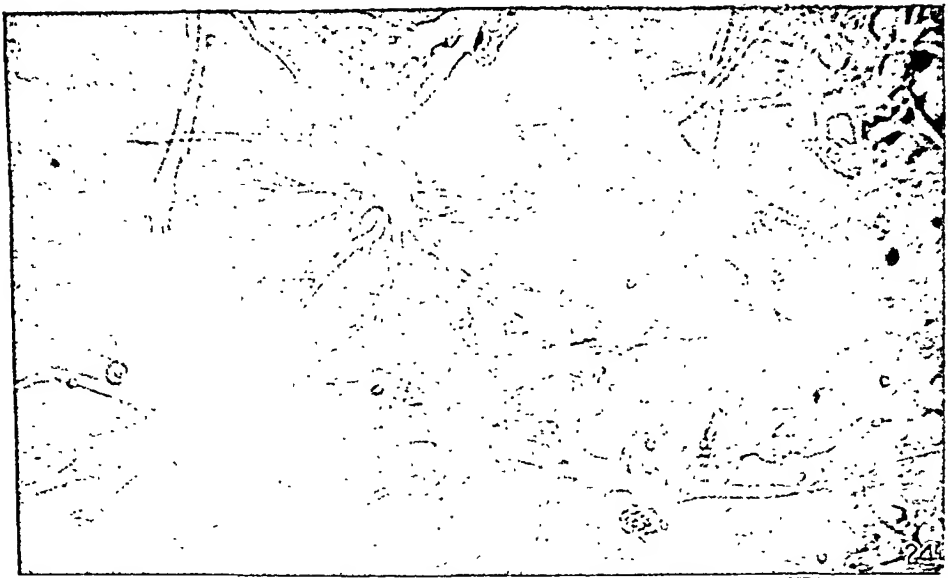
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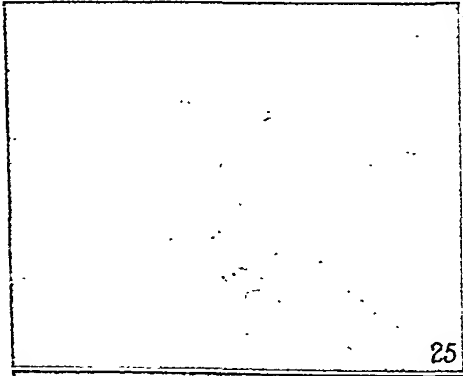
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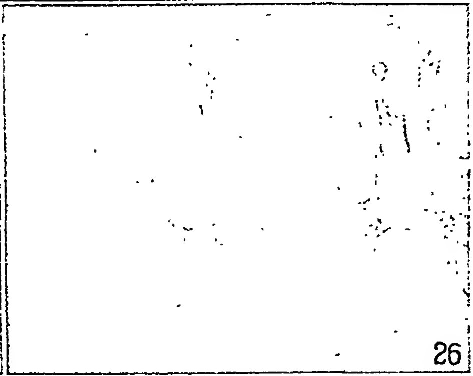
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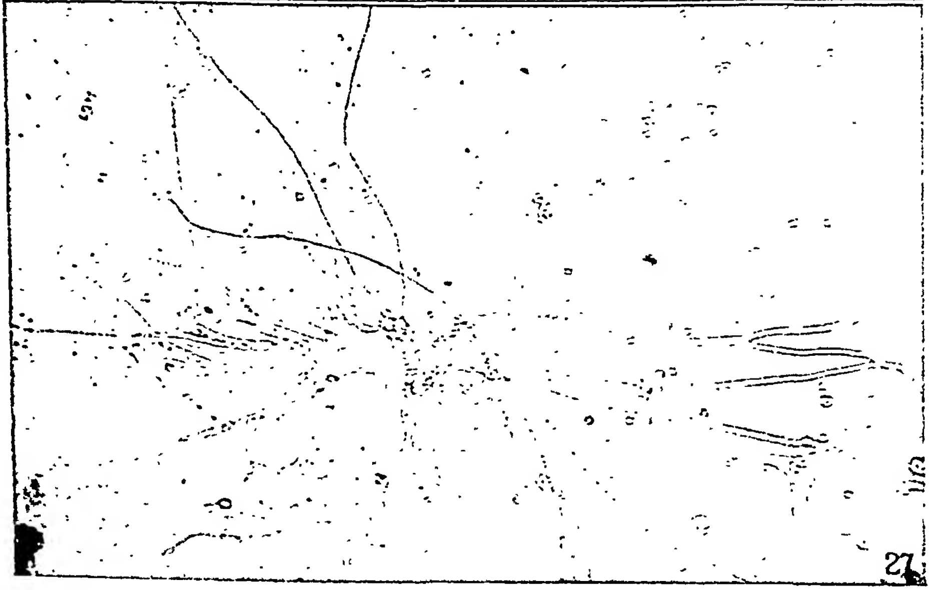
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(Parker: Races constituting common fibroblasts. II)

STUDIES ON THE PREVENTION OF CHOLESTEROL ATHEROSCLEROSIS IN RABBITS

I. The Effects of Whole Thyroid and of Potassium Iodide

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For 25 years the atherosclerosis produced in herbivores by feeding cholesterol or cholesterol-rich foods has attracted the attention of investigators. The literature, already of formidable proportions, has been recently reviewed by Anitschkov (1). The work to date has been concerned chiefly with the production and study of the lesions. Our interest has been in their prevention.

The rabbit as an experimental animal for the study of aortic disease is viewed with suspicion by some workers, largely because of their failure to distinguish clearly between intimal and medial involvement of the aorta.

The lesions produced by feeding cholesterol are intimal, appearing as elevated, yellowish plaques most commonly in the arch, ascending aorta, and around the mouths of the intercostal arteries. When extensive, the process often involves the aortic valve. Calcification of the plaques may occur. The accumulation of large, fat-laden cells beneath the endothelium is a prominent histologic feature. By some pathologists these intimal lesions are considered the analog of human atherosclerosis.

On the other hand, medial lesions of the rabbit's aorta have been produced by a variety of agents such as adrenalin, barium chloride, thyroxin, viosterol, bacterial products, nicotin, and many other substances. The process is characterized by a degeneration of the muscle cells in the media often followed by a deposition of calcium. The intima is involved secondarily if at all.

The importance of distinguishing sharply between these two types of involvement is due to the high frequency of spontaneous medial degeneration, while spontaneous intimal lesions are rare. In fact it is doubtful whether the latter ever occur in young rabbits.

Control Observations

Before studying the prevention of atherosclerosis it was necessary to be certain that an effective method for the production of these intimal lesions was being employed.

The rabbits used throughout this work were mainly of the Dutch belted variety, although a few rabbits of other breeds were necessary to fill in groups from time to time. The animals were from 4 to 6 months old at the beginning of each experiment. They were kept in individual cages indoors under similar conditions, and were fed a diet consisting of oats, an alfalfa-molasses mixture, and fresh vegetables. As the work has been in progress practically continuously since Oct., 1930, it was possible to rule out a seasonal effect and none, in fact, was noted.

The method of administration of cholesterol favored by most investigators has been to give the substance dissolved in a warm vegetable oil by stomach tube. This method was tried at first but soon abandoned because several rabbits developed inhalation pneumonias, while others stopped eating normally after the oil. It was finally decided to mix 1 gm. of crystalline cholesterol with each rabbit's grain three times a week. The disadvantages of this method are its wastefulness and a lack of knowledge as to the exact cholesterol intake. The advantages, however, are its simplicity, freedom from trauma, lack of interference with normal eating habits, and—not the least consideration—its effectiveness.

In the first test of this method, a group of 15 young rabbits was used. Seven of these were kept as controls and received only a regular diet. Eight rabbits were given cholesterol, at first by stomach tube, later simply mixed with the grain.

In a second group were 16 rabbits, also 4 to 6 months old. Eight of these were used as controls, and 8 were given cholesterol as above. In addition each of the 16 animals had 5 drops of viosterol 250D (Squibb) instilled into the pharynx three times a week. It was thought that viosterol in this dosage might hasten or increase the severity of the changes produced by cholesterol. In so far as aortic lesions, whether intimal or medial, were concerned, the viosterol appeared to be entirely inert. Accordingly, for convenience of consideration, it seemed justifiable to combine the first and second groups. The results were as follows:

	No. of rabbits	Duration of experiment	Cholesterol intake*	Atherosclerosis	
				Present	Absent
		days	*gm.*		
Controls..........	15	92–113	0	0	15
Cholesterol-fed....	5	9– 53	2–10	0	5
	11	87–114	25–37	11	0

* It must be emphasized that figures given for cholesterol dosage represent the maximal possible intake. Due to the limitations of the feeding method used, it is not only possible but probable that the actual intake was less than the amount given in each instance.

No atherosclerosis was found in the aortas of the 15 control rabbits either grossly or microscopically.

It has long been recognized that a time factor is important in the production of the lesions that occur after cholesterol feeding. In the cholesterol-fed group 5 rabbits died in 9 to 53 days, having received approximately 2 to 10 gm. of cholesterol. There was no atherosclerotic change in the aortas. The remaining 11 animals were killed after 87 to 114 days and a cholesterol intake of 25 to 37 gm. Slight to marked macroscopic atherosclerotic change was present in the aorta of each of the 11 rabbits. Involvement of other arteries was also noted in most of the animals together with characteristic changes in such organs as the liver, spleen, kidneys, and adrenals, but, as these lesions have been adequately described by previous investigators and as they do not fall within the restricted scope of the present report, detailed references to them will be omitted.

As the result of this preliminary work it was evident that gross atherosclerosis could be produced in the aortas of 4 to 6 month old rabbits in approximately 90 days by simply mixing crystalline cholesterol with the grain. It is probable that the lesions may appear in a shorter time, but a minimal survival period of 90 days was arbitrarily established as a requirement for inclusion in the series that follow.

Effect of Whole Thyroid and of Thyroxin

In view of the clinical observation that the blood cholesterol tends to be low in hyperthyroidism with a corresponding tendency to abnormally high levels in myxedema, it was decided to try the effect of thyroid given concurrently with cholesterol upon the production of atherosclerosis. It was further decided to test the efficacy both of a whole gland product and of thyroxin.

Control Group: Cholesterol-Fed.—This group contained 21 rabbits from 4 to 6 months old. Cholesterol was added to the diet as in the preliminary work.

Seven animals died in 45 to 83 days and were discarded. Three of these had atherosclerotic lesions at 57, 69, and 83 days respectively.

Fourteen rabbits were killed after 90 to 118 days. The results are given in Table I. Gross atherosclerotic lesions of the aorta were present in every case. A determination of the whole blood cholesterol by the method of Bloor, Pelkan, and Allen (2) was made in 11 rabbits from blood obtained shortly before the animal was killed. Cholesterol values in the blood ranged from 326 to 790 mg. per 100 cc., and averaged 520 mg. By the same method the blood cholesterol of 25 normal rabbits of a comparable age was found to average 105 mg. with a range of 82 to 169 mg.

TABLE I

Cholesterol-Fed Rabbits Used as Controls for Thyroid Series

Rabbit No.	Sex	Weight		Duration of experiment	Cholesterol intake	Blood cholesterol*	Atherosclerosis of aorta	Remarks
		Start	End					
		kg.	*kg.*	*days*	*gm.*	*mg. per 100 cc.*		
A1-4	F.	1.3	1.8	90	35	381	Moderate	
A1-0	M.	1.7	1.8	98	38	532	Marked	
A1-1	M.	1.8	1.8	98	38	326	Slight	
4-6	M.	2.2	2.0	108	39	—	Marked	
4-8	F.	1.8	1.8	108	39	—	Marked	
A7	F.	1.5	1.7	113	44	397	Slight	
A8	F.	2.1	2.4	113	44	543	Marked	
A1	M.	1.7	1.8	114	44	423	Moderate	O_2 consumption per gm. per hr. 0.472 cc. (average)
A2	M.	1.4	1.7	114	44	545	Marked	
A3	F.	1.5	1.8	114	44	588	Moderate	
A4	F.	1.9	2.1	114	44	790	Marked	
A5	M.	1.6	1.9	114	44	484	Slight	
A6	F.	1.8	2.1	114	44	714	Marked	
5-0	M.	1.6	2.1	118	39	—	Marked	
Average..............................						520		

* In a series of 25 normal rabbits the average blood cholesterol was found to be 105 mg. with a range from 82 to 169 mg.

The Effect of Whole Thyroid.—Thirty rabbits were used in this group. Each animal received approximately 0.4 gm. of dried whole thyroid (Parke, Davis) as a powder together with 1 gm. of cholesterol mixed with the grain three times a week. Again it must be pointed out that, as in the case of the cholesterol, the dosage represents the maximal possible intake. The actual amount ingested was almost certainly somewhat less. The rabbit exhibits a surprising tolerance to thyroid given by mouth. Weight loss, however, was usually marked in this series. One lot of animals was practically wiped out by a protracted period of hot weather that occurred during the thyroid feeding, and it was necessary to intermit the feeding of the survivors (Nos. 7-3, 7-4, 7-5).

Eleven rabbits in the group died in 30 to 77 days, having received 11 to 28 gm. of cholesterol and 5.2 to 10.8 gm. of thyroid. No gross atherosclerosis was present. These animals were excluded from the series.

TABLE II

Effect of Whole Thyroid upon Production of Atherosclerosis by Cholesterol

Rabbit No.	Sex	Weight		Duration of experiment	Cholesterol intake	Thyroid intake	Blood cholesterol	Atherosclerosis of aorta	Remarks
		Start	End						
		kg.	*kg.*	*days*	*gm.*	*gm.*	*mg. per 100 cc.*		
7-3	F.	1.9	1.3	90	28	10.8	—	0	
7-4	F.	1.7	1.4	90	28	10.8	—	0	
7-5	F.	1.9	1.3	90	28	10.8	—	0	
A2-4	F.	1.7	1.3	90	35	14.0	—	0	Died; hyperthyroid?
A1-8	M.	1.9	1.1	93	36	14.4	—	0	Died; hyperthyroid?
A2-1	M.	1.7	1.2	95	37	14.8	205	0	
A2-8	M.	1.6	1.1	95	37	14.8	140	0	
A3-1	F.	1.9	1.1	95	37	14.8	114	0	
A2-7	F.	1.7	1.4	98	38	15.2	291	Slight	
A1-5	F.	1.9	1.3	100	39	15.6	261	Moderate	O_2 consumption per gm. per hr. 0.799 cc. (average)
A1-6	M.	1.6	1.4	100	39	15.6	103	0	
A2-2	M.	1.8	1.1	100	39	15.6	205	0	
A1-7	F.	1.1	0.9	107	42	16.8	104	0	
A1-9	M.	1.9	1.5	107	42	16.8	147	0	
A2-5	F.	1.6	1.2	107	42	16.8	154	0	
A3-0	F.	1.7	1.2	107	42	16.8	132	0	
A2-0	M.	1.9	1.2	108	43	17.2	202	0	
A2-3	M.	1.4	1.4	108	43	17.2	167	0	
A2-6	F.	1.7	1.2	108	43	17.2	263	0	
Average							178		

Nineteen animals were killed or died in 90 to 108 days. The results are shown in Table II. The cholesterol intake varied between 28 and 43 gm., and the thyroid from 10.8 to 17.2 gm. Atherosclerosis of the aorta was absent in 17 rabbits, while gross lesions were present in 2. In 1 of these (No. A2-7) the process was limited to a few small plaques in the descending thoracic aorta. The other rabbit (No. A1-5) showed widespread thickening of the aortic intima, but the plaques were usually discrete and did not coalesce.

The whole blood cholesterol, determined in 14 animals shortly before they were killed, ranged from 103 to 291 mg. per 100 cc. with an average of 178 mg. Both rabbits with atherosclerotic plaques in the aorta had a hypercholesterolemia. One animal also had a considerably elevated blood cholesterol (263 mg.) but no aortic lesions.

The Effect of Thyroxin.—Each rabbit in the preceding group received a maximum of 1.2 gm. of whole thyroid per week. This contained 1.4 mg. of thyroxin. It was desirable to give each animal in the present group an equivalent amount of thyroxin. However, it was decided to give 1.2 mg. instead of 1.4 mg. because the whole amount was to be injected subcutaneously, and because the thyroid-fed rabbits probably did not eat all of the dried gland that was mixed with their food. Therefore the slightly smaller dose seemed more nearly equivalent.

TABLE III

Effect of Thyroxin upon Production of Atherosclerosis by Cholesterol

Rabbit No.	Sex	Weight		Duration of experiment	Cholesterol intake	Thyroxin intake	Blood cholesterol	Atherosclerosis of aorta	Remarks
		Start	End						
		kg.	*kg.*	*days*	*gm.*	*mg.*	*mg. per 100 cc.*		
A3-3	M.	1.4	1.0	90	35	16.8	317	Slight	
6-3	M.	2.7	2.0	94	29	12.0	—	Marked	
A3-9	M.	1.8	1.3	98	38	16.8	368	0	
A3-2	F.	1.7	1.7	107	42	19.2	381	Marked	
A3-6	M.	1.6	1.7	107	42	19.2	170	0	
A3-8	M.	1.7	1.4	107	42	19.2	348	Moderate	
6-1	M.	2.4	1.7	108	39	13.2	—	Moderate	
6-4	F.	2.1	1.7	108	39	13.2	—	0	
A4-0	M.	2.1	1.6	112	44	19.2	658	Moderate	
A4-1	M.	1.6	1.3	112	44	19.2	379	Marked	
A4-2	M.	1.8	1.4	112	44	19.2	568	Moderate	O_2 consumption per gm. per hr. 0.626 cc. (average)
Average							399		

Accordingly, each rabbit in this series was given the usual gram of cholesterol three times a week, and once a week received a subcutaneous injection of 1.2 mg. of thyroxin (Squibb) dissolved in N/50 NaOH.

Of the 17 animals comprising this group, 6 died in 44 to 80 days and were excluded from the series. The aortas of 5 of these rabbits were normal. The sixth rabbit, dying after 44 days and having received 17 gm. of cholesterol and 7.2 mg. of thyroxin, showed early, diffuse intimal lesions.

Eleven rabbits were killed in 90 to 112 days. The results are shown in Table III. Eight had slight to marked atherosclerotic changes in the aorta. The remaining 3 animals had normal aortas both grossly and microscopically.

The blood cholesterol in 8 rabbits varied from 170 to 658 mg., and averaged 399 mg. All the animals with aortic lesions had a hypercholesterolemia. Rabbit A3-9 had a blood cholesterol of 368 mg. but a normal aorta. This, in our experience, is an unusual combination. Another rabbit (No. A3-6) without atherosclerosis had a relatively normal blood cholesterol of 170 mg.

Discussion.—The facts presented in Tables I, II, and III may be summarized as follows:

Group	No. of rabbits	Atherosclerosis		Average blood cholesterol
		Present	Absent	
				mg.
Cholesterol	14	14	0	520
Cholesterol + thyroid	19	2	17	178
Cholesterol + thyroxin	11	8	3	399

The effectiveness of whole thyroid when given simultaneously with cholesterol in preventing the atherosclerosis and hypercholesterolemia caused by the latter is striking. The comparative ineffectiveness of thyroxin is equally marked. There is no obvious explanation for this difference in response, although the opportunity it affords for speculation is abundant.

A similar effect of thyroid was noted in 1918 by Murata and Kataoka who, in the transactions of the Japanese Pathological Society, reported that they had observed that the experimental arteriosclerosis produced by lanolin feeding was absent or slight in degree when thyroid feeding was combined with the lanolin (3). No amplification of this brief statement has been found.

Another point of interest has to do with oxygen consumption. This was determined on 1 animal in each of the 3 groups through the kindness of Dr. Dickinson W. Richards, Jr. In spite of a substantial elevation of the basal oxygen consumption of both Rabbits A1-5 (Table II) and A4-2 (Table III), *i.e.* a good thyroid effect, gross atherosclerosis of the aorta was present, and the blood cholesterol was 261 and 568 mg. respectively.

Effect of Potassium Iodide

Potassium iodide has long been used empirically in patients with arteriosclerosis and has occasionally seemed of real benefit. Therefore it was decided to try the effect in a group of rabbits of giving potassium iodide simultaneously with cholesterol.

Because the efficacy of the cholesterol feeding method in causing the appearance of the aortic lesions had already been demonstrated it seemed permissible to use a smaller control group than had been employed previously. Seven rabbits were set aside as controls and were given a gram of cholesterol three times a week according to the usual method. Unfortunately only 3 of these animals survived the requisite period of 90 days. These 3 rabbits were killed on the 98th day, having received 39 gm. of cholesterol. The blood cholesterol was 658, 694, and 472 mg. respectively. Atherosclerosis was evident in the aorta of each animal. In 1 it was classed as moderate in degree, while in 2 it was marked. Atherosclerotic changes were also present in 2 rabbits dying in 49 and 78 days respectively. In 2 other animals dying at 39 and 40 days no lesions were present.

TABLE IV

Effect of KI upon Production of Atherosclerosis by Cholesterol

Rabbit No.	Sex	Weight		Duration of experiment	Cholesterol intake	KI intake	Blood cholesterol	Atherosclerosis of aorta
		Start	End					
		kg.	*kg.*	*days*	*gm.*	*gm.*	*mg. per 100 cc.*	
A7-9	F.	1.8	2.1	99	39	39	197	0
A8-0	M.	2.1	2.1	99	39	39	154	0
A8-1	M.	1.5	1.8	99	39	39	138	0
A8-2	F.	1.9	1.8	99	39	39	185	0
A8-3	F.	2.0	2.1	99	39	39	135	0
A8-4	F.	1.4	1.6	100	39	39	240	0
A8-5	F.	2.2	1.9	100	39	39	248	Marked
A8-6	M.	2.1	1.9	100	39	39	136	0
A8-7	M.	1.5	2.0	100	39	39	255	0
A4-4	F.	1.6	1.8	112	44	44	169	0
A4-5	M.	1.5	1.6	112	44	44	111	0
A4-6	M.	2.3	2.3	113	44	44	231	0
Average							183	

The Effect of KI.—Twelve rabbits were fed a gram of cholesterol and a gram of potassium iodide—the latter in aqueous solution—three times a week. The animals in this group were killed after 99 to 113 days (Table IV). Atherosclerosis of the aorta was absent in 11, and present in only 1. It was, however, marked in this instance. The blood cholesterol varied between 111 to 255 mg. with an average of 183 mg. In 4 animals over 200 mg. of cholesterol per 100 cc. of blood were present, and in 3 of these no atherosclerosis was found. The only rabbit in the series to develop atheromata had a blood cholesterol of 248 mg.

The Effect of KBr and KCl.—To determine, if possible, whether the inhibiting effect of potassium iodide upon the development of the atherosclerosis produced by cholesterol was due to the potassium ion or to the iodine, and to rule out a non-specific halogen action, a group of rabbits was fed with cholesterol and potassium bromide and a second group was given cholesterol and potassium chloride.

TABLE V

Effect of KBr upon Production of Atherosclerosis by Cholesterol

Rabbit No.	Sex	Weight		Duration of experiment	Cholesterol intake	KBr intake	Blood cholesterol	Atherosclerosis of aorta
		Start	End					
		kg.	*kg.*	*days*	*gm.*	*gm.*	*mg. per 100 cc.*	
A5-9	F.	1.4	1.6	100	39	39	424	Marked
A6-2	F.	1.2	1.7	100	39	39	297	0
A6-4	F.	1.3	1.7	100	39	39	431	Marked
A6-5	M.	1.5	1.6	100	39	39	431	Marked
A6-7	M.	1.8	2.1	100	39	39	325	Marked
A6-0	M.	1.6	1.2	101	39	39	293	Marked
A6-8	F.	1.6	1.2	101	39	39	798	Moderate
Average							428	

TABLE VI

Effect of KCl upon Production of Atherosclerosis by Cholesterol

Rabbit No.	Sex	Weight		Duration of experiment	Cholesterol intake	KCl intake	Blood cholesterol	Atherosclerosis of aorta
		Start	End					
		kg.	*kg.*	*days*	*gm.*	*gm.*	*mg. per 100 cc.*	
A7-6	M.	1.5	1.4	99	39	39	—	Moderate
A6-9	F.	2.0	2.0	101	39	39	511	Marked
A7-1	M.	1.6	1.9	101	39	39	490	Moderate
A7-2	F.	1.3	1.6	101	39	39	403	Marked
A7-3	F.	2.3	2.2	101	39	39	765	Marked
A7-4	F.	1.5	1.9	102	39	39	309	Moderate
A7-5	F.	1.4	1.9	102	39	39	544	Marked
A7-7	M.	1.6	1.8	102	39	39	119	0
A7-8	F.	1.6	1.8	102	39	39	284	Moderate
A8-9	M.	1.4	1.6	102	39	39	436	Moderate
Average							429	

The results obtained with potassium bromide feeding are shown in Table V.

Each rabbit received the usual gram of cholesterol three times a week together with a gram of KBr in aqueous solution. Of the 7 rabbits in the group, 6 developed gross atherosclerosis. These animals showed a blood cholesterol ranging from 293 to 798 mg. The seventh animal had a normal aorta, macroscopically and microscopically, although a hypercholesterolemia was also present. The average blood cholesterol for the group was 428 mg.

There were 10 rabbits in the group given cholesterol and potassium chloride (Table VI). Nine developed atherosclerosis. All had a hypercholesterolemia of from 284 to 765 mg. The one animal in this group that did not show atherosclerosis was the only one with a normal blood cholestérol. The average value for the cholesterol in the blood in the 9 rabbits in which it was determined, was 429 mg.

Discussion.—The effects of KBr, KCl, and KI upon the atherosclerosis produced in the aorta of rabbits by feeding cholesterol may be summarized as follows:

Group	No. of rabbits	Atherosclerosis		Average blood cholesterol
		Present	Absent	*mg.*
Cholesterol	3	3	0	608
Cholesterol + KBr	7	6	1	428
Cholesterol + KCl	10	9	1	429
Cholesterol + KI	12	1	11	183

The effectiveness of potassium iodide in preventing cholesterol atherosclerosis when administered simultaneously with the cholesterol is apparent. That this is due to the iodine and not to the potassium and that it is not a general halogen effect is suggested by the inadequacy of both the bromide and chloride.

Liebig (4) was the first to report a retarding influence of iodine upon cholesterol atherosclerosis in rabbits. Using an organic iodine preparation he at first was able to demonstrate no effect. Later, after increasing the amount of iodine administered, he was able to prevent the development of lesions in 3 of the 4 rabbits used. Subsequently (5) he stated that he had increased his series to 28 animals and that in 75 per cent he found either no lesion or only slight lesions in the aorta. No protocols were given.

Seel and Creuzberg (6) fed 3 rabbits cholesterol and egg yolk for 30 days. The serum cholesterol showed a prompt rise, with a fall to normal within 3 weeks after cholesterol was stopped. The animals were killed later and it was stated that more or less atherosclerosis was found in the aortas. Three other rabbits were given cholesterol in the same manner for 30 days and then given an organic iodine preparation. There was no atherosclerosis when the rabbits were autopsied later. Finally, to 3 rabbits cholesterol and then potassium iodide were given. Aortic lesions were absent in 2, present in 1. Because of the importance of the time factor in the development of atheromatous lesions after cholesterol feeding, this work fails to be convincing, as 30 days seem too short a time to ensure the presence of aortic changes.

SUMMARY

1. Whole thyroid gland when administered simultaneously with cholesterol prevented the atheromatous changes produced by the latter in the aorta of rabbits in 17 of 19 animals.
2. In this series thyroxin was less effective, as atherosclerosis occurred in 8 of 11 rabbits.
3. Potassium iodide also exerted a strong protective action as aortic lesions were present in only 1 of a series of 12 rabbits fed cholesterol and potassium iodide concurrently.
4. The effectiveness of potassium iodide was not shared by potassium bromide or potassium chloride.
5. A relationship was noted between the level of the cholesterol in the blood and the development of atherosclerosis. In general, the aortic lesions accompanied a hypercholesterolemia.

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STUDIES ON THE PREVENTION OF CHOLESTEROL ATHEROSCLEROSIS IN RABBITS

II. The Influence of Thyroidectomy upon the Protective Action of Potassium Iodide

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In a previous paper (1) it was reported that both whole thyroid and potassium iodide were effective in preventing the atherosclerosis and hypercholesterolemia produced by feeding rabbits cholesterol. The mechanism of this protective action was obscure. Because of the prominent place occupied by the thyroid gland in the metabolism of iodine it seemed an obvious and logical step to remove the thyroid and to determine the effect of this procedure upon the efficacy of potassium iodide administered to these animals simultaneously with cholesterol.

Dutch belted rabbits were used together with a few of a mixed breed. The animals were about 4 or 5 months old at the beginning of the experiment. They were divided into 3 groups. About half of the animals in each group were thyroidectomized. A midline incision was made over the trachea. The thyroid was exposed and dissected out as completely as possible. Care was taken to avoid removing the external parathyroids. This operation is a comparatively simple procedure in young rabbits, and it is borne well by them.

All of the animals were kept indoors, in individual cages, and were fed a stock diet of oats, an alfalfa and molasses mixture, and fresh vegetables. The thyroidectomized animals were apt to eat poorly for a week or two after the operation and lost weight during this period. Thereafter they ate normally and gained back the weight lost although they did not tend to become obese.

A blood cholesterol determination was made on each rabbit every 10 days by the method of Bloor, Pelkan, and Allen (2). Blood studies upon the thyroidectomized rabbits were not started as a rule until 2 weeks after the operation when the animals were again eating normally. Observations were continued for as long as 110 days.

Group I. Control Rabbits.—In this group there were 5 normal rabbits and 7 that had been thyroidectomized. They were given the regular diet without the addition of cholesterol. The results are shown in Tables I and II.

Although rather marked variations appeared in the blood cholesterol values in animals of the same group and in the same animal at different times, the general level was about the same for both the normal and the thyroidectomized rabbits. If anything, it was slightly higher for the latter.

No atherosclerosis of the aorta was found in any rabbit of this group. In two instances a slight amount of thyroid regeneration had occurred, presumably from failure to effect complete removal of the upper poles at operation.

TABLE I

Normal Animals. Regular Diet

Rabbit No.	Sex	Weight		Blood cholesterol, *mg. per 100 cc.*												Atherosclerosis of aorta	Remarks
				Days													
		Start	End	1	10	20	30	40	50	60	70	80	90	100	110		
		kg.	*kg.*														
1-31	F.	1.8	2.5	123	134	134	128	144	117	117	99	139	94	114	103	0	Killed, day 110
1-32	M.	1.9	2.1	111	155	115	90	119	110	99	105	90	98	104	94	0	Killed, day 110
1-33	M.	1.6	1.9	92	135	113	91	115	120	92	81	95	92	80	102	0	Killed, day 110
1-34	M.	1.7	1.9	89	125	98	80	103	144	98	93	114	94	98	94	0	Killed, day 110
1-35	F.	1.7	2.5	124	208	156	107	117	123	90	94	126	97	85	99	0	Killed, day 110
Average				108	151	123	99	120	123	99	94	113	95	98	98		

Group II. Rabbits Fed Cholesterol.—There were 5 normal and 7 thyroidectomized rabbits in this group. All received the stock diet to which was added a gram of cholesterol per animal three times a week. The results are given in Tables III and IV.

Four of the intact animals showed a prompt, pronounced rise in blood cholesterol by the 20th day. One of these died on day 42 and had no aortic lesions, as was to be expected after so short a feeding period. Atherosclerosis was present in the aortas of the other 3 at 110 days.

The fifth intact rabbit did not at any time show as marked a hypercholesterolemia as did the others. Despite this it developed atherosclerotic lesions. This is the only instance we have encountered of a rabbit with a normal or only slightly elevated blood cholesterol developing atherosclerosis of the aorta.

All of the thyroidectomized rabbits showed a prompt rise in blood cholesterol of approximately the same magnitude, however, as that of the normal animals fed cholesterol. Three of the 7 rabbits died in 38 to 59 days and, at autopsy, were free from atherosclerotic changes in the aorta. One rabbit died on day 79 and had moderate aortic atherosclerosis. The remaining 3 animals were killed after 110 days. These all showed atherosclerosis. Thyroid regeneration had occurred to a slight degree in 1 rabbit.

TABLE II

Thyroidectomized Animals. Regular Diet

Rabbit No.	Sex	Weight		Blood cholesterol, *mg. per 100 cc.*												Atherosclerosis of aorta	Remarks
				Days													
		Start	End	1	10	20	30	40	50	60	70	80	90	100	110		
		kg.	*kg.*														
1-14	M.	1.2	1.8	98	135	170	168	141	112	137	134	92	169	93	106	0	Killed, day 110. Some thyroid regeneration
1-15	M.	2.0	2.5	128	170	155	136	115	108	117	104	98	123	98	104	0	Killed, day 110. Some thyroid regeneration
1-18	M.	2.0	2.4	92	98	117	125	113	119	122	103	90	98	107	90	0	Killed, day 110. Thyroids out
1-19	F.	1.8	2.2	90	178	195	184	135	177	125	105	104	156	146	94	0	Killed, day 110. Thyroids out
1-20	F.	2.1	2.0	87	—	131	151	130	103	130	159	—	—	—	—	0	Died, day 74
1-23	F.	1.7	2.3	98	125	191	208	105	154	134	—	—	—	—	—	0	Transferred to Group II
1-26	M.	1.9	2.2	96	80	101	122	134	90	94	101	101	90	82	104	0	Killed, day 110. Thyroids out
Average				99	131	151	156	125	123	123	118	97	127	105	100		

Group III. Rabbits Fed Cholesterol and KI.—Six normal and 8

TABLE III

Normal Rabbits Fed Cholesterol

Rabbit No.	Sex	Weight		Blood cholesterol, *mg. per 100 cc.*												Atherosclerosis of aorta	Remarks
				Days													
		Start	End	1	10	20	30	40	50	60	70	80	90	100	110		
		kg.	*kg.*														
1-36	M.	1.7	2.1	101	267	302	268	338	344	375	318	368	457	395	535	Marked	Killed, day 110
1-37	F.	2.1	2.5	128	133	288	297	384	—	—	—	—	—	—	—	0	Killed, day 42
1-38	F.	1.8	2.4	109	206	446	625	978	1013	853	961	893	852	765	937	Marked	Killed, day 110
1-39	F.	2.2	2.3	111	156	375	208	432	551	514	469	399	390	599	493	Marked	Killed, day 110
1-40	M.	2.2	2.5	85	117	184	150	164	188	147	150	170	216	218	179	Slight	Killed, day 110
Average............				107	176	319	310	459	524	472	475	458	479	494	536		

TABLE IV

Thyroidectomized Rabbits Fed Cholesterol

Rabbit No.	Sex	Weight		Blood cholesterol, *mg. per 100 cc.*												Atherosclerosis of aorta	Remarks
		Start	End	Days													
				1	10	20	30	40	50	60	70	80	90	100	110		
		kg.	*kg.*														
1-03	M.	1.7	2.2	105	226	260	235	320	284	234	354	260	347	412	416	Marked	Killed, day 110. Thyroids out
1-04	F.	1.0	1.4	169	288	416	649	568	639	853	750	669	—	—	—	Moderate	Died, day 79. Pneumonia. Thyroids out
1-05	F.	1.3	1.4	121	268	260	288	507	—	—	—	—	—	—	—	0	Killed, day 38. Diarrhea. Thyroids out
1-06	M.	1.7	2.1	85	156	215	250	293	383	436	—	—	—	—	—	0	Died, day 59. Pneumonia. Thyroids out
1-08	M.	0.9	0.8	160	196	234	426	568	617	—	—	—	—	—	—	0	Died, day 55. Pneumonia. Thyroids out
1-09	F.	2.1	2.3	121	131	288	493	536	721	625	528	668	734	646	886	Marked	Killed, day 110. Slight thyroid regeneration
1-23	F.	2.2	2.3	148	303	481	493	521	543	481	493	650	493	536	507	Moderate	Killed, day 110. Thyroids out
Average				130	224	308	405	473	531	526	531	562	525	531	606		

thyroidectomized rabbits were used in this group. Each animal received the regular diet to which was added a gram of cholesterol and a gram of potassium iodide in aqueous solution three times a week.

The 6 normal rabbits in this group fed cholesterol and potassium iodide survived for 110 days (Table V) at which time they were killed. No atherosclerosis could be demonstrated. This agrees with the observation made in our previous work (1), that potassium iodide administered simultaneously with cholesterol prevents the atherosclerosis regularly produced by the latter. The blood cholesterol values of these 6 rabbits were similar in general to those of the animals in the control group, although the averages tended to run somewhat higher particularly in the later days of the experiment.

TABLE V

Normal Rabbits Fed Cholesterol and KI

Rabbit No.	Sex	Weight		Blood cholesterol, *mg. per 100 cc.*												Atherosclerosis of aorta	Remarks
				Days													
		Start	End	1	10	20	30	40	50	60	70	80	90	100	110		
		kg.	*kg.*														
1-41	M.	1.3	1.6	150	250	187	175	183	175	254	268	147	148	199	288	0	Killed, day 110
1-42	M.	1.9	2.1	170	208	191	228	163	191	229	276	212	234	268	279	0	Killed, day 110
1-43	M.	1.6	2.0	134	170	180	—	139	104	103	134	159	147	143	187	0	Killed, day 110
1-44	M.	1.7	2.1	114	94	187	118	110	97	124	145	144	156	113	134	0	Killed, day 110
1-45	F.	1.4	1.5	165	144	144	146	204	125	187	141	228	195	204	288	0	Killed, day 110
1-46	M.	1.7	1.7	142	117	122	163	90	90	128	156	169	162	115	135	0	Killed, day 110
Average.......				146	164	168	166	148	130	171	187	177	174	174	218		

It is of interest that an additional rabbit, not included in the table, that had had only one lobe of the thyroid removed at operation, behaved in all respects similarly to the intact animals. Its blood cholesterol at the beginning was 170 mg., and after 110 days, during which it averaged 186 mg., the final value was 184 mg. At autopsy no atherosclerosis was present. This suggested that the protective effect of the iodide could be maintained after the removal of half the thyroid tissue.

The 8 thyroidectomized rabbits (Table VI) all showed a rise in blood

TABLE VI

Thyroidectomized Rabbits Fed Cholesterol and KI

Rabbit No.	Sex	Weight		Blood cholesterol, *mg. per 100 cc.*												Atherosclerosis of aorta	Remarks
				Days													
		Start	End	1	10	20	30	40	50	60	70	80	90	100	110		
		kg.	*kg.*														
A7-0	F.	1.8	—	163	252	338	528	500	625	—	—	—	—	—	—	Slight	Died, day 56. Thyroids out
W8-6	M.	—	—	96	293	288	—	—	—	—	—	—	—	—	—	—	Experiment stopped, day 30
A9-7	M.	—	—	91	185	197	213	334	—	—	—	—	—	—	—	—	Experiment stopped, day 43
1-12	F.	1.4	1.9	156	268	417	367	407	399	354	624	781	750	852	797	Marked	Killed, day 110. Thyroids out
1-24	F.	1.9	2.3	97	288	264	367	375	383	268	382	383	347	375	408	Moderate	Killed, day 110. Thyroids out
1-29	F	1.7	1.9	128	235	521	481	—	—	—	—	—	—	—	—	0	Died, day 41. Pneumonia. Thyroids out
1-48	M.	1.2	1.0	125	284	—	—	—	—	—	—	—	—	—	—	0	Died, day 19. Pneumonia. Thyroids out
1-52	M.	1.7	1.9	80	179	170	170	159	218	307	—	347	375	399	399	Moderate	Killed, day 110. Thyroids out
Average				117	248	314	354	355	406	310	503	582	491	542	535		

cholesterol comparable to that occurring in the animals in Group II fed cholesterol without potassium iodide. In 2 rabbits, however, this rise was somewhat delayed.

The administration of potassium iodide and cholesterol was discontinued in 2 animals, as will be mentioned later, after the rise in blood cholesterol had occurred. Two rabbits died in 19 and 41 days respectively. No atherosclerosis was found. One died after 56 days. Early diffuse atherosclerotic changes were present. Three animals survived 110 days and were then killed. All showed atheromatous lesions in the aorta. No thyroid regeneration occurred in the rabbits of this group.

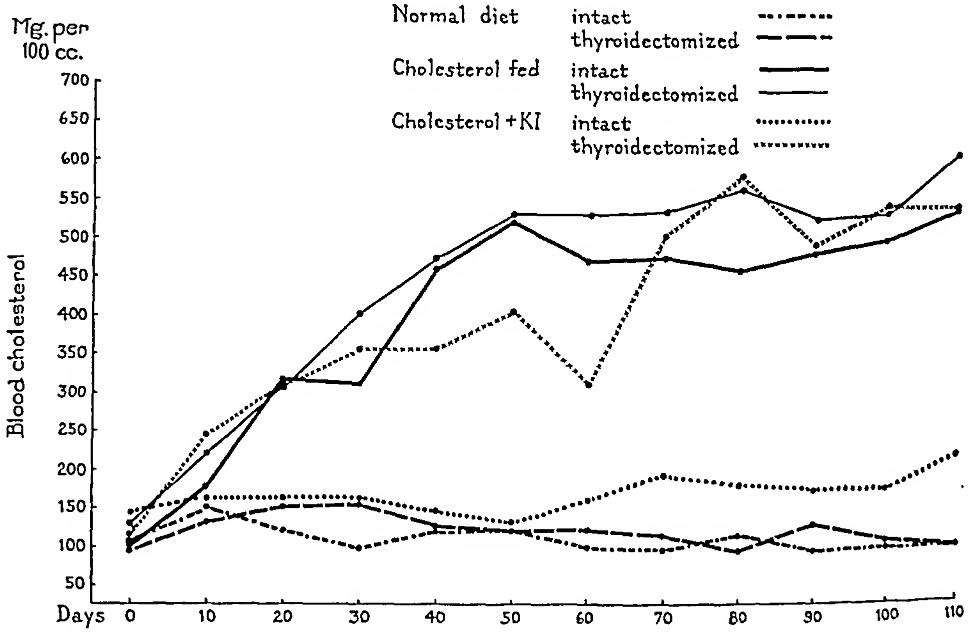

FIG. 1. Curves representing the average blood cholesterol values are given for both the intact and thyroidectomized rabbits in the 3 experimental groups over a period of 110 days. The important fact is demonstrated that the blood cholesterol of normal intact rabbits fed cholesterol and KI approximates that of the control group, while the blood cholesterol of thyroidectomized animals fed cholesterol and KI parallels that of the group fed cholesterol alone.

The average blood cholesterol values of the 3 groups, normal and thyroidectomized, are plotted for the 110 day period in Fig. 1. The important fact that this figure demonstrates is that the blood cholesterol of normal intact rabbits fed cholesterol and potassium iodide follows closely that of the control group; while the cholesterol in the blood of the thyroidectomized animals fed cholesterol and potassium iodide parallels that of the group fed cholesterol alone.

Two rabbits (Nos. W8-6 and A9-7), mentioned above, deserve further comment. A thyroidectomy was performed on these rabbits, following which they were given potassium iodide and cholesterol for 30 days in one case and 43 days in the other. Both responded with a prompt rise in blood cholesterol (Table VI). The administration was then stopped for a period of approximately 6 months during which the animals were given a normal diet. The cholesterol and potassium iodide were again resumed and continued for 110 days. This time, in contradistinction to the first period of feeding, no significant rise in the blood cholesterol occurred. In other words, these thyroidectomized animals reacted in the manner of intact rabbits fed cholesterol and potassium iodide. The reason for this was apparent when the animals were autopsied. Each showed a mass of vascular, regenerated thyroid tissue as large as or larger than the normal thyroid in size. Probably this regeneration had occurred during the period in which the feeding was interrupted and had progressed to such a degree that, when the experiment was resumed, the amount of thyroid tissue was adequate for the protective action of potassium iodide to take place.

CONCLUSIONS

1. Thyroidectomy in itself does not cause a rise in blood cholesterol or the development of atherosclerosis in young rabbits.
2. Feeding cholesterol produces hypercholesterolemia and atherosclerotic lesions in rabbits regardless of the presence or absence of the thyroid glands.
3. Potassium iodide prevents the usual hypercholesterolemia and atherosclerosis of the aorta in normal rabbits fed cholesterol, but when the thyroids are removed this protective action disappears.

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QUANTITATIVE STUDIES ON THE PRECIPITIN REACTION

ANTIBODY PRODUCTION IN RABBITS INJECTED WITH AN AZO PROTEIN*

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The antibody titer of a serum has usually been given in terms of the highest dilution at which it will agglutinate, hemolyze, or precipitate the antigen, in terms of the volume of toxin it will neutralize, in terms of the optimal proportion in which flocculation with antigen takes place, in terms of mouse protection—all relative and often inaccurate measures giving no idea whatsoever of the actual mass of antibody involved. The dilution methods, particularly, are subject to an error of 50 to 100 per cent in the decision as to which tube is the last positive one.

For the quantitative measurement of precipitins one need no longer be dependent upon such methods. With the recognition of antibodies as modified serum globulins (1) and with the establishment by the writers of the conditions for their maximum precipitation (2) an absolute method for the estimation of precipitating antibodies has been worked out (3), based on the earlier use, by Wu and his coworkers (4), of the micro Kjeldahl method for the analysis of antigen-antibody precipitates. The first application of this method was in the standardization of Type I antipneumococcus horse sera (3), in which the writers, with Sia, showed a parallel between mouse protection and the maximum amount of specifically precipitable protein, a relation which was confirmed by Felton (5). A preliminary report was then made on the amounts of antibody produced following injection of a red azo

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dye, R-salt-azo-benzidine-azo-crystalline egg albumen (6). Culbertson has also used the method in a study of the crystalline egg albumen-antibody system and devised a modification suitable for that system (7). The method may also be reversed, and used for the micro determination of precipitating haptens such as specific polysaccharides (8), or for antigens. A similar method for the analysis of antigen-antibody precipitates has been employed by Marrack and Smith (9), while the optimal proportions method has been proposed both for the determination of antigen (10) and of antibody (11).

In the quantitative determination of antibody in an antigen-antibody system it is necessary to distinguish between two kinds of protein, since both antigen and antibody are, as far as is known, proteins. This problem was first solved in two ways by Wu and his collaborators (4) by using hemoglobin and iodoalbumen as antigens and determining their amount in the specific precipitate by appropriate methods. Unfortunately, Wu's studies extended over only a portion of the reaction range, and at the time the conditions for the maximum precipitation of antibody were not understood.

To the writers it seemed that the simplest way of distinguishing between antigen and antibody nitrogen (or protein) would be to employ colored antigens such as the azo proteins which had proved so valuable in the study of the chemical basis of specificity by Landsteiner (12) and by Avery and Goebel (13). For the purpose of a quantitative study of the relations between antigen and antibody in the precipitin reaction it was felt that a crystalline protein, such as egg albumen, should be used, and that it should be coupled with a more intensely colored dye than had been found necessary by these workers, in order that colorimetric determination of the antigen in precipitates or supernatants might be as accurate as possible. It was also felt that the azo antigen should be freed from components showing the original egg albumen specificity in order to eliminate the necessity of using a second azo antigen in the quantitative precipitin tests. A purplish red disazo dye, R-salt-azo-benzidine-azo-egg albumen, was finally isolated in a form which satisfied these requirements and was used in the quantitative studies on antibody production referred to above, and in a study of the mechanism of the precipitin reaction (6). Since this preliminary report azo proteins have been found useful in quantitative work by Marrack and Smith (14) and by Breinl and Haurowitz (15).

The quantitative studies on the mechanism of the precipitin reaction between R-salt-azo-benzidine-azo-egg albumen and its homologous antibody will be reported in detail in a separate communication. The present paper deals with quantitative observations on precipitin production in rabbits following multiple injections of known amounts of the azo antigen.

EXPERIMENTAL

1. Preparation of the Azo Protein.—0.46 gm. of benzidine was dissolved in 100 cc. of water containing 3 cc. of 1:1 hydrochloric acid and tetrazotized at 7–8°C. with an aqueous solution of 0.35 gm. of sodium nitrite. The solution was poured into a chilled solution of 3 gm. of sodium acetate in 500 cc. of water, and to this was added a solution of 0.87 gm. of R-salt in 100 cc. of water. The R-salt was an especially pure preparation supplied through the kindness of Dr. M. L. Crossley of The Calco Chemical Company. The mixture slowly reddened, but the coupling was not completed until 20 cc. of normal potassium carbonate solution had been added. The intensely colored solution now contained tetrazotized benzidine coupled on one side with R-salt, but with the other diazo group free.

Three times recrystallized egg albumen (16) was dialyzed free from ammonium salts and 6 gm. of the protein in 1 liter of water at room temperature were made alkaline with 40 cc. of 2 N potassium carbonate solution. 100 cc. of the above diazo solution were then run in every 10 to 20 minutes as tests made by adding a few drops of the solution to carbonate-containing R-salt solution showed coupling to be complete. After a total of 600 cc. of diazo solution had been run in 20 cc. more of 2 N potassium carbonate solution were added. While egg albumen is capable of combining with somewhat more diazo solution than the amount actually used, it was considered advisable not to continue the coupling process to the limit, as in other cases this had resulted in insoluble complexes. Potassium carbonate was used since potassium salts of the diazo component and the coupled protein dye appeared to be less easily salted out at the above concentrations than did the sodium salts.

The dye protein solution was next chilled and acidified with acetic acid until flocculation first occurred, the optimum pH range varying from 4.6 to 4.2 in different preparations. The bicolor standard method was used (17), adding blank tubes of the pink supernatant. In this way good readings could be obtained with bromocresol green. The main supernatant, on acidification with more acetic acid, yielded less highly colored material, and this was either discarded or added to a subsequent preparation.

The crude R-salt-azo-benzidine-azo-egg albumen was collected by centrifugation, dissolved in about 750 cc. of water with the minimum amount of N sodium carbonate solution, centrifuged to remove a small amount of violet-colored insoluble material, and again acidified with the minimum amount of acetic acid required for flocculation.[1] In this way small amounts of less highly colored protein, reactive with anti-egg albumen serum, generally remained in solution, so that under optimal conditions 20 to 25 repetitions of the process resulted in recovery of the main portion of the azo protein as a clear, purplish red solution which no longer precipitated anti-egg albumen serum.[2]

In order to remove non-protein dye impurities the mixture was centrifuged as sharply as possible after the fifth or sixth precipitation and stirred in a freezing mixture with chilled acetone for ½ hour. After centrifugation (always in the cold) the dark red supernatant was discarded and the precipitate taken up in cold water and redissolved and reprecipitated as before. The acetone treatment usually resulted in denaturation of a small portion of the dye.

In one preparation 17 reprecipitations with acid sufficed to reduce the content of substances reacting with anti-egg albumen serum so that solutions as strong as 0.2 per cent failed to precipitate the serum. However, sera of rabbits immunized with the dye are precipitated by suitable concentrations of egg albumen. Quantitative experiments have indicated that it is not anti-egg albumen, but antibody to the dye which is precipitated by egg albumen from these sera. The details will be presented separately.

In another preparation 25 reprecipitations failed to remove impurities reactive with anti-egg albumen serum. The solution was then adjusted to 0.5 per cent phenol concentration and allowed to stand over the summer in the ice box, freezing solid during the period. After the ice had been melted it was found that much of the azo protein had been denatured. The entire mixture was diluted to 1.5 liters, stirred for 2 hours at room temperature after addition of 75 cc. of N sodium carbonate solution, stirred 2 hours longer in the cold, and was finally centrifuged. The precipitate was again extracted with very dilute sodium carbonate solution and the insoluble residue was discarded. The extracts were precipitated with acetic acid and solution and reprecipitation repeated five times, after which the dye, when redissolved, no longer reacted with anti-egg albumen serum. It was also possible in this case to sterilize the solution by filtration through a Berkefeld V candle, a process which had been unsuccessfully attempted with the preceding batch.

As a final step, the dye solution was ultrafiltered in the ice box through a collodion membrane and repeatedly washed with sterile 0.9 per cent saline. The washings were colorless at first, owing to adsorption of the dye by the porous earthenware support of the membrane, but eventually much colored material passed through and this was not precipitable by acetic acid or by antiserum to the dye. Washing was discontinued when the color of the filtered solution became

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[1] All operations were conducted in the cold, and a refrigerating centrifuge, manufactured by the International Equipment Company, Boston, was used.

[2] Four sera of high antibody content, kindly supplied by Dr. J. T. Culbertson, yielded traces of precipitate after centrifugation in the cold.

less intense than that of a 1:100,000 solution of the dye. The filter contents were then centrifuged to remove traces of violet precipitate and the dye solution was standardized by analysis for nitrogen by the micro Kjeldahl method. The azo protein was found to contain 14.6 per cent of nitrogen on the ash-free basis. The solution was kept sterile in the ice box by the occasional addition of a drop of chloroform.

2. Immunization of Rabbits with the Azo Protein.—In all but one series the injections were given intravenously. Four daily injections were given each week for 4 weeks, after which bleedings were made on the day indicated. Many animals were given additional courses of 2, 3, or 4 weeks. In only a few instances were more than 2 or 3 mg. injected at one time. The procedure used for each group of animals is summarized in the tables.

Material for the experiments summarized in Table II was obtained as follows: 1.5 cc. of a solution of the ultrafiltered azo protein containing 13.8 mg. were diluted to 10 cc. and treated with 0.08 cc. of 0.1 N acetic acid, an amount insufficient to precipitate the dye but adequate to increase the amount adsorbed by collodion particles over the amount taken out in neutral solution. The dye solution was rinsed into a suspension of collodion particles which had been prepared in the usual way by dilution of an acetone solution of collodion with water and rejection of the coarse particles. The mixture was stirred occasionally during several hours, allowed to stand overnight in the ice box, and was then centrifuged. The supernatant was poured into a volumetric flask and to it were added the next two washings with water. A third washing with saline extracted no more dye. The amount of dye left in the supernatant was determined colorimetrically by comparison with a solution of known concentration, giving, by difference, the amount adsorbed on collodion. The suspension was then diluted with saline (plus 0.01 per cent merthiolate) so that 50 cc. of suspension, the amount used for 16 injections, contained 0.55 mg. of dye. The initial dose was 1.0 cc. and this was gradually increased, so that at the end the rabbits each received 5.0 cc. Eight animals were injected in this way. The 50 cc. of suspension contained 140 mg. of collodion.

The remaining animals which received smaller amounts of collodion-adsorbed dye were in earlier series.

For comparison with the eight animals receiving 50 cc. of the collodion suspension, five rabbits were treated with identical doses of a *solution* of the dye at the same dilution, and five more with a *suspension* of the same concentration containing 14 per cent by volume of 1 per cent alum solution (18).

In the last three lines of Table II are summarized the data on a number of rabbits injected with stronger solutions and more concentrated alum suspensions of the azo protein.

3. Determination of the Maximum Amount of Precipitable Antibody in the Sera.—In the original method (3) the precipitate formed by the specific polysaccharide of Type I pneumococcus and its homologous antibody was washed only once with a 1:20,000 solution of specific polysaccharide in saline. It has since been found (8) unnecessary to add the specific carbohydrate to the washing fluid, nor does dissociation occur in the azo protein-antibody system when the precipitate is washed in the cold with 0.9 per cent saline alone. Two washings are also necessary (8) if complete removal of non-specific serum proteins is desired. The method follows as applied in the present instance:

Depending on the intensity of a preliminary rapid qualitative test with 1:10,000 dye, amounts of serum ranging from 0.5 to 4.0 cc. are used. A number of sera may be analyzed at one time. The sera should be measured in duplicate with accurately calibrated pipettes into wide agglutination tubes (10 mm. inside diameter x 75 mm.) or Wassermann tubes, depending on the amount of serum used. Blanks should also be run in duplicate and saline added to these instead of dye solution. If less than 2 cc. of serum have been used the volume should be made up to 2 cc. with saline. An amount of a 1:1000 solution of the dye in saline is then added sufficient to provide a *slight* excess of the dye. Not more than 0.10 cc. should be added in the case of sera containing less than 0.1 mg. of precipitable antibody per cc. (3 to 4 cc. samples), and the volume of stronger sera should be chosen so that not more than 0.2 or 0.3 cc. of the 1:1000 dye solution need be used. Calibrated pipettes are not necessary for the dye. The tubes are plugged and the contents carefully and thoroughly mixed by a rotary motion imparted by drawing the fingertips rapidly and repeatedly diagonally down the side of the tube. The tubes are set in the water bath and may be centrifuged, if desired, as soon as the precipitate begins to settle, in order to make sure that an excess of dye has been added. If the supernatant is not definitely pink as compared with a blank on the same serum, 0.05 to 0.1 cc. more of the dye dilution should be added at a time until a definite excess is present. Supernatants should not contain so large an excess as to be definitely red, as many sera show a marked inhibition zone beginning with surprisingly low concentrations of antigen in excess. If the supernatants are red, more serum should be added to the determinations and blanks, or a new analysis started. In the case of weak sera the precipitates are often very slow in forming. The tubes are allowed to stand 2 hours in the water bath at 37° and overnight in the ice box, or else may be left at room temperature for a period and then overnight in the ice box, or may be immediately put into the ice box for 15 to 18 hours. The rabbit antisera tested in this laboratory have given identical results under these conditions, except that the precipitate forms at a slower rate in the cold. The systems tested have been dye-antidye, egg albumen and its homologous antibody, and Type III pneumococcus specific polysaccharide and rabbit anti-Type III pneumococcus serum, so that in these cases there would seem to be no basis, except on the ground of increased speed of reaction, for the current immunological practice of allowing precipitin tests to stand 2 hours at 37° before placing in the ice box overnight. This observation does not apply to immune horse sera, in which the differences found are being subjected to closer study.

After the tubes have stood overnight they are centrifuged in the refrigerating centrifuge or in carriers containing ice water for 15 to 20 minutes at about 1500 revolutions per minute. The supernatants are then carefully decanted and the tubes are inverted, allowed to drain 5 minutes, and the mouths wiped with filter paper. The tubes are placed in ice water and 0.5 cc. of ice-cold saline is added to each. The contents are mixed as before and the red precipitates should be thoroughly disintegrated in order to insure as complete removal of non-specific protein as possible. The tubes are then rinsed down with 1.5 cc. of ice-cold saline in the case of the small tubes and 2.5 cc. for the Wassermann tubes and again mixed.

Blank tubes which show no whirl when the contents are mixed at the first or second washing may be discarded, as under these conditions the blank to be deducted in the nitrogen determination is no larger than that on the reagents alone.

While the tubes are standing in ice water for ½ hour the original supernatants should be tested as a control for the presence of a slight excess of dye. To one 0.5 cc. portion of the mixed duplicate supernatants from each serum is added 0.1 cc. of a 1:10,000 dye solution, to another, 0.2 to 0.3 cc. of the blank supernatant from a serum which has given a heavy precipitate. There should, of course, be no precipitate in the tube to which additional dye was added, while the tube to which antiserum was added should show a slight turbidity within 2 hours or at least a slight precipitate on standing overnight in the ice box.

After ½ hour in the cold the washed precipitates are centrifuged, decanted, and drained as before, and again washed as above with 1.5 or 2 cc. of chilled saline, depending on the size of tube. After standing for ½ hour in ice water, the tubes are finally centrifuged, decanted, and drained. The precipitates are covered with 1.5 to 2 cc. of water, loosened from the bottom of the tube by rotating as before, and dissolved by the addition of 2 to 3 drops of N sodium hydroxide solution.

The amount of azo protein in the precipitate may be determined by making up the volume of the solution to 5.0 or 10.0 cc. and comparing the color with that of a known solution of the dye containing the same amount of alkali and a few milligrams of added colorless protein. Since the ratio of dye to antibody in the precipitate has been found in this laboratory to average 1:7 at the equivalence point (for definition see (2, 6, 1 *b*)), the determination of antigen in the precipitate may be omitted if it is desired to accept this figure. In the application of the method to any other antigen it would be necessary to determine this ratio for the system used (*cf.*, for example, (7)).

The solution of the precipitate is rinsed quantitatively into a micro Kjeldahl flask and the nitrogen determined by any standard procedure. The Pregl method, slightly modified, was used in the present work. Nitrogen found × 6.25 = specifically precipitated protein in the sample. Total protein minus antigen protein $\left(\frac{\text{total}}{8}\right)$ = antibody. For the determination of the total amount of circulating antibody in the animal the blood volume was taken as 5.5 per cent of the weight at the time of bleeding, according to Meek and Gasser (19), and the serum volume as one-half of the blood volume.

DISCUSSION

1. Precipitin Content of Rabbit Sera as Influenced by the Period between Final Injection and Bleeding.—In Table I is given a summary of antibody determinations on sera taken at various periods after the final injection of azo protein. 3 days was the shortest interval and 10 days the longest. It is apparent that the maximum antibody content

TABLE I

Influence of Days between Last Injection and Bleeding, also of Repeated Bleedings, on Precipitin Content of Rabbit Sera

Rabbit No.	Total antigen injected	Total No. of injections	Weight precipitable antibody per cc. on day indicated after last injection						
			3rd	4th	5th	6th	7th	8th	10th
	mg.		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
	In solution								
5-5	18.7	18*				0.97		0.75	
6-8	13.2	16				1.22	1.04		
2nd course									
5-5	26.4	25*	0.79	0.61	0.39				
5-6	26.4	25*	0.36	±†	±†				
6-2	26.4	25*	0.83	0.85	0.47				
6-4	20.9	23	0.66	0.62					
6-8	20.9	23	2.06	1.88	2.04				
6-9	20.9	23	1.09	0.88	1.08				
3rd course									
5-5	53.9	32	±		0.79		0.81		0.89
6-1	53.9	32	±		0.94		0.86		0.80
6-2	53.9	32	±		0.42		0.36		
	As alum precipitate								
8-9	14.5	18	2.03	1.91					
1-03	36.1	19			1.43	1.48			
1-12	28.4	32			0.95		0.75		
1-15	19.1	30			1.87 (test bleeding)				
					1.58 (main bleeding, same day)				
1-20	28.1	16			0.46		0.43		
	On collodion particles								
1-31	0.55	16			0.24		0.24		
1-33	0.55	16			0.54		0.23		

* All but two injections subcutaneous.

† Traces of precipitate in the 0.5 cc. samples used.

was usually reached by the 3rd day after the last injection. In one series, in which unusually large doses of 6.7 mg. had been given at the end, the sera yielded only traces of precipitate on the 3rd day.

It is also clear that with these exceptions and in the case of one 10th day bleeding in the same series[3] of animals, the maximum titer obtainable was contained in the first bleeding. In subsequent bleedings within 1 or 2 days different animals varied greatly in the antibody content of the serum yielded, some showing great constancy, and others a gradual or irregular diminution. In one case (No. 1-15) the serum from a small initial bleeding of not more than 5 cc. contained 1.89 mg. of precipitable antibody per cc., while the serum from the main bleeding, only several hours later, contained but 1.58 mg. per cc.

As a result of these tests it is the custom in this laboratory to bleed animals 5 or 6 days after the last injection, although any day from the 4th to the 10th would probably serve as well.

2. *Experiments Summarized in Table II.*—All of the 18 rabbits injected intravenously with 0.55 mg. of the azo antigen in multiple doses either in solution, as an alum precipitate, or adsorbed on collodion particles, responded with measurable amounts of precipitin. Four rabbits which received as little as 0.35 mg. on collodion particles also responded well. The lower limit for the amount of azo protein capable of stimulating precipitin formation appears to be about 0.2 mg. in multiple doses, for of 10 rabbits receiving 0.15 to 0.23 mg. on collodion particles, only two showed precipitins. This is in accord with the findings of Hektoen and Cole (20), who placed the lower limit for egg albumen at about 0.3 mg.

The magnitude of the antibody response to multiple minimal doses of the antigen is indeed surprising. It has, of course, long been known in a qualitative way that antibody production is in excess of the amount required to combine with the antigen, and this fact provided Ehrlich with a strong argument against the Buchner hypothesis of antibody formation. Quantitative data on this point are now made available in Table II, from which it is seen that as much as 0.73 to

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[3] Most of the analyses in this series and the one preceding it were carried out on 0.5 cc. samples in order to keep the individual bleedings as small as possible. The difference between the 5th and 10th day bleedings is therefore within the limit of error on so small a sample of a serum of comparatively low titer.

0.94 mg. of circulating antibody per cc. of serum may be formed in response to injections of antigen totaling 0.35 to 0.55 mg., or a total response for the rabbit of over 100 mg. of circulating antibody for every milligram of antigen injected. This is at least 12 times as much as is necessary to combine with the amount of antigen used. There is also an appreciable amount of antibody in the tissues as well, and the presence of antibodies other than precipitins is not excluded. Thus the relative mass of antibody formed is so large as to be taken as supplementing other recent evidence (1*b*) against Buchner's hypothesis of the actual entrance of specific antigen fragments into the antibody molecule.

TABLE II

Precipitin Formation in Rabbits Following Multiple Injections of R-Salt-Azo-Benzidine-Azo-Crystalline Egg Albumen

No. of rabbits	Total dye injected into each	Total No. of injections	Precipitable antibody per cc.	Total circulating precipitin	Maximum amount circulating precipitin formed per mg. injected
	mg.		*mg.*	*mg.*	*mg.*
5	0.55 (solution)	16	0.06–0.53	4–44	80
5	0.55 (alum)	16	0.28–0.73	25–51	93
8	0.55 (collodion particles)	16	0.07–0.86	5–60	109
4	0.35 (collodion particles)*	16	0.16–0.73	10–38	109
4	0.23 (collodion particles)	11	0, 0, 0, 0.04	0–3	13
4	0.15 (collodion particles)	14	0, 0, 0, 0.10	0–8.5	57
	Experiments with Larger Doses				
5	0.55† + 3.1 (alum)	22	0.51–1.53	29–109	30
23	9.2 – 36.1 (alum)	16–33	0, 0, 0, 0.17–3.39	0–203	21
10	12.9 – 26.4 (solution)	13–25	0, 0, 0.50–2.08	0–129	6

* After three more injections with an additional 0.1 mg. of dye on collodion particles the serum of the best animal contained 0.94 mg. of precipitable antibody per cc., or a total of 49 mg. for the entire circulating precipitin (No. 1-14, Table IV).

† This group consisted of the five best antibody producers among 15 rabbits in the first three groups.

A summary is also given in Table II of precipitin formation in 10 rabbits which received larger doses of the azo protein in solution, and in two series totalling 28 rabbits which received the dye as the alum precipitate. The total amounts of azo protein injected ranged from 3.7 to 35 mg., and it is seen that while the maximum relative response is lower with the larger doses, the total amount of precipitin produced is often higher. As stated by Hektoen (21) there is no relation between the dosage and the amount of precipitin produced.

Sixteen sera contained more than 1 mg. of precipitin per cc. Thirteen of these were in the alum group, as was the serum of highest titer; namely, 3.39 mg. per cc. This serum contained 75 mg. of protein per cc. (calculated from total nitrogen), so that in this rabbit 4.5 per cent of the serum protein consisted of antibody.[4]

Five of the rabbits, of which three were in the alum group, failed to show qualitative or quantitative evidence of circulating precipitins, further emphasizing the enormous individual variations in the immunological response of rabbits. In spite of these individual differences it would seem fair to give the preference to multiple intravenous injections of the dye as the alum precipitate, since sera of higher antibody content were obtained more regularly when the dye was used in particulate form. The use of the dye adsorbed on collodion particles is naturally limited to very small doses.

After the final bleeding one rabbit (No. 1-57) which had received 0.55 mg. of the dye adsorbed on collodion particles and one which had received 35.1 mg. of the alum precipitate (No. 1-32) were autopsied. The spleens and portions of the livers were fixed in bichloride-acetic acid solution and unstained sections were made. These were kindly examined by Dr. Franklin M. Hanger, Jr., of this Department, who found, in the first animal, collodion particles in the Kupffer cells and in the phagocytic cells of the reticulum of the spleen. In the second animal azo protein particles could be seen in a few of the same groups of cells (*cf.* also (22)).

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[4] The highest antibody titer observed in this laboratory occurred in the serum of a rabbit given repeated small intravenous injections of formalinized Type III pneumococcus vaccine. The amount of protein specifically precipitable from this serum by the Type III pneumococcus specific polysaccharide was 17.0 mg. per cc. Calculated from the total nitrogen, the serum contained 91 mg. of protein per cc. Thus more than one-sixth of the serum protein was anticarbohydrate. Antipneumococcus sera titering 7 to 9 mg. per cc. of anticarbohydrate were frequently encountered (see also (3)).

The ability of a rabbit to build up antibodies to the azo protein failed to serve as an index of the animal's response to stimulation with another antigen such as Pneumococcus or Streptococcus. A number of excellent antibacterial sera were obtained from rabbits which had given antidye sera of low precipitin content. Conversely, Rabbits 6-8 and 6-9 yielded antidye sera of average precipitin content but gave very little anticarbohydrate when subsequently injected with Pneumococcus III.

TABLE III

Precipitin Content of Rabbit Sera as Influenced by Repeated Courses of Injections of Antigen Solution

Rabbit No.	Course of injections	Total antigen injected	Total No. of injections	Precipitable antibody per cc.	Day of bleeding
		mg.		*mg.*	
5-5	1	18.7	18	0.97	6
	2	26.4	25	0.79	3
	3	53.9	32	0.89	10
5-6	1	18.7	18	0.55	6
	2	26.4	25	0.36	3
6-4	1	13.2	16	0.62	6
	2	20.9	23	0.66	3
6-8	1	13.2	16	1.22	6
	2	20.9	23	2.06	3
	3	53.9	32	0.52	5
	4	88.0	48	2.30	4
6-9	1	13.2	16	0.77	6
	2	20.9	23	1.09	3
	3	53.9	32	0.39	5
5-2	1	18.7	18	0.63	6
	2	26.4	25	±*	5
6-2	1	18.7	18	±*	6
	2	26.4	25	0.85	4

* Traces of precipitate.

3. *Variations in Precipitin Content of Sera of Rabbits Given More than One Course of Injections.*—The data are summarized in Tables III

and IV. For the sake of brevity only the principal types of behavior observed are recorded. Perhaps the most frequently encountered types of variation were (1) that in which the rabbit reached a relatively low but maximum antibody content with the first course, and (2) a precipitin content which increased with successive courses to a maximum. For most rabbits there appeared to be a maximum number of

TABLE IV

Precipitin Content of Rabbit Sera as Influenced by Repeated Courses of Injections of Azo Protein in Particulate Form

Rabbit No.	Course of injections	Total antigen injected	Total No. of injections	Precipitable antibody per cc.	Day of bleeding
		mg.		*mg.*	
1-03	1	0.23 (collodion particles)	11	0.04	6
	2	36.1 (alum)	19	1.48	6
	3	74.4 (alum)	32	1.43	5
1-04	1	0.23 (collodion particles)	11	0	6
	2	36.1 (alum)	19	±[*]	6
	3	76.5 (alum)	33	0.52	3
1-14	1	0.35 (collodion particles)	16	0.73	4
	2	0.45 (collodion particles)	19	0.94	4
	3	9.2 (alum)	25	3.39	5
	4	19.1 (alum)	33	3.33	5
1-33	1	0.55 (collodion particles)	16	0.54	5
	2	3.6 (alum)	22	0.51	6
	3	6.2 (alum)	29	0.51	6
	4	8.9 (alum)	37	0.33	6
1-40	1	0.55 (alum)	16	0.71	5
	2	3.6 (alum)	22	1.48	6
	3	6.2 (alum)	29	0.36	6
	4	8.9 (alum)	37	0.77	6
1-46	1	0.55 (solution)	16	0.53	5
	2	3.6 (alum)	22	1.53	6
	3	6.2 (alum)	29	1.53	6
	4	8.9 (alum)	37	1.42	6

[*] Traces of precipitate.

milligrams of precipitin which the individual could produce as a result of stimulation with repeated doses of the azo protein, whether the amount used in a single dose were a small fraction of a milligram or as high as 8 mg.

4. *Stability of Antisera.*—In Table V are given the original precipitin content of a number of sera and the number of milligrams found per cubic centimeter after periods up to 9 months in the refrigerator. The tubes were covered with rubber caps. Sera 1-12 to 1-20 contained 0.01 per cent of merthiolate. Only in the case of three sera was the loss in precipitin outside the limits of error of the method. It appears to be a widespread belief that antiprotein sera rapidly lose their antibody content, but the antisera to the azo protein in question certainly afford no support for this belief. Several of the sera are being preserved in sealed tubes and it is hoped to analyze them after a longer period.

TABLE V

Influence of Storage on the Precipitable Antibody of Rabbit Sera

Rabbit No.	Weight precipitable antibody per cc. at time of bleeding	Interval between determinations	Precipitable antibody per cc. after storage
	mg.	*mos.*	*mg.*
6-0	1.06	7	0.99
6-8	2.04	5.5	1.48
6-9	1.08	5.5	1.04
8-9	1.92	4.5	1.42
9-3	0.83*	9	0.83
1-12	0.75	6	0.67
1-14	3.33	6	3.06
1-15	1.59	6	1.53
1-16	1.02	6	0.99
1-17	0.65	6.5	0.59
1-18	0.70	6.5	0.76
1-19	1.29	6.5	1.21
1-20	0.43	6.5	0.40

* This analysis was made 3 months after the bleeding.

SUMMARY

1. The preparation is described of a deep red protein dye, R-salt-azo-benzidine-azo-crystalline egg albumen, which contains no more than traces of protein with the original egg albumen specificity.

2. Based on previous publications of the writers, a quantitative method is given for the micro estimation of precipitin in the antisera to the dye. The method gives the actual weight of precipitin and may be applied to the determination of the maximum amount of precipitable antibody in any antiserum.

3. Data are given (1) on the influence of the period between the final injection and the bleeding on the precipitin content of rabbit antisera to the azo protein; (2) on the magnitude of the antibody response following the injection of multiple doses of the antigen varying within wide limits; (3) on the variations in the precipitin content of the sera of rabbits given successive courses of antigen injections; and (4) on the stability of antisera stored in the cold.

4. Four antisera were obtained in which over 100 times as much precipitin was recovered as the amount of antigen injected. This supplements the growing mass of evidence against the theory that specific antigen fragments are actually incorporated into the antibody molecule.

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8-9	1.92	4.5	1.42
9-3	0.83*	9	0.83
1-12	0.75	6	0.67
1-14	3.33	6	3.06
1-15	1.59	6	1.53
1-16	1.02	6	0.99
1-17	0.65	6.5	0.59
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THE EFFECT OF PNEUMOCOCCUS AUTOLYSATES UPON PNEUMOCOCCUS DERMAL INFECTION IN THE RABBIT

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The injection of virulent pneumococci into the skin of a rabbit's flank gives rise to an edematous lesion which spreads ventrally until the entire midabdominal area of the skin is involved (1). Previous studies (2) have shown that the edema fluid may be drained from this lesion, and that, if taken during the earlier phases of the infection, this fluid not only fails to clot but has the property of retarding the coagulation of normal rabbit blood. The origin of the antithrombic property of this edema fluid was not definitely proven but it was shown that pneumococcus autolysates also possessed a similar property and the suggestion was made that the bacteria might be the source of the anticoagulant substance in the edema fluid. It is not unreasonable to suspect that some of the less well protected microorganisms may be damaged in the earlier phases of the pneumococcus infection, and that the resulting death and dissolution of these less resistant bacterial cells may take place with the liberation of autolytic products which, by retarding coagulation and altering vascular permeability, may promote the invasion of the surviving organisms.

In a continuation of the study of this problem, the experiments to be reported in this paper were undertaken to determine whether bacterial autolysates might promote the invasiveness or enhance the virulence of pneumococci. Although in the course of these experiments a variety of different strains of pneumococci of various specific types have been studied, only the results obtained with Pneumococcus Type III are included in the present paper, since strains of this type exhibiting different degrees of virulence for rabbits offer excellent opportunity for the study of infections under selective and experimentally reproducible conditions.

EXPERIMENTAL

Cultures.—The strains used for infection were as follows:

Pneumococcus Type III (Strain PH), virulent for both mice and rabbits.

Pneumococcus Type III (Strain A66), highly virulent for mice but never leading to the death of rabbits when injected intravenously or intradermally.

Pneumococcus (Strain M 3 R), rough organism, derived from a strain of Type III Pneumococcus. Avirulent for both mice and rabbits.

Autolysates.—The bacteria from 18 hour broth cultures were collected by centrifugation and suspended in amounts of saline equivalent to 1 per cent of the original culture volume. The bacterial suspensions were quickly frozen and thawed eight times and then allowed to stand in the ice box for 1 week. A suitable preparation of autolyzed Pneumococcus produces purpura in mice when injected intraperitoneally in 0.5 cc. amounts, and, when added to freshly drawn rabbit blood, causes a marked prolongation of clotting time. Before injection, all bacterial autolysates were heated at 70°C. for 15 minutes to insure the death of all viable pneumococci.

Infections.—Rabbits were infected intradermally by the method previously described (1). Varying amounts of sterile autolysate and living culture were mixed and immediately injected into the skin of the flank area of rabbits.

The use of the dermal method of infection permits a thorough observation of the progress of the infective process and allows the differentiation of what is commonly called virulence into (*a*) lethal capacity and (*b*) invasiveness, the latter meaning capacity to provoke infection, as indicated by the production of a local lesion, and the stimulation of non-fatal systemic reactions. Ordinarily in experimental work, it is customary to refer to virulence as the ability of the microorganism to bring about the death of the infected animal, but a strict application of the term virulence must also include the capacity of an organism to invade the tissues without causing death, since there are obviously many organisms capable of inciting a disease which under ordinary conditions is seldom if ever fatal.

Enhancement of Virulence of a Virulent Strain

Experiments were designed to determine whether pneumococcus autolysates enhance the virulence of a strain which when injected alone may produce disease and death in rabbits. The strain of Type III Pneumococcus designated PH usually produces a fatal dermal infection in rabbits when injected in amounts as small as 0.000,01 cc. of broth culture, but never causes death when less than this amount of culture is used. Varying amounts of this culture together with fixed quantities of pneumococcus autolysates derived from the same or other strains were injected intradermally in the flank areas of rabbits.

Table I shows the results of such an experiment in terms of survival and death of the infected rabbits.

In this instance, the culture alone brought about death of the rabbit when 0.000,1 cc. was injected. Animals receiving amounts of culture less than this survived. On the other hand, when mixed with 0.2 cc. of an autolysate of the homologous strain, 1/10,000 the lethal number of the same organism sufficed to induce a fatal infection. Colony counts showed that the number of pneumococci contained in 0.000,000,01 cc. of the culture was less than 10. Thus the virulence has been so enhanced that whereas with the culture alone approximately 10,000 pneumococci were required to bring about a fatal infection, less than 10 organisms caused death when injected together with autolysate.

TABLE I

Effect of Autolysates on the Infective Capacity of a Strain of Pneumococcus Virulent for Rabbits

Culture: Rabbit-virulent Type III Pneumococcus (Strain PH). Autolysates and culture mixed at time of injection. All injections intradermal.

Amount of broth culture	Materials added to pneumococcus culture			
	0.2 cc. of saline (control)	0.2 cc. of autolysate homologous strain (PH)	0.2 cc. of heterologous autolysate (Pneumococcus Type I)	0.2 cc. of autolysate of an R strain of Pneumococcus derived from Type III
cc.				
0.000,000,01	—	D	S	S
0.000,000,1	—	D	D	D
0.000,001	S	D	D	D
0.000,01	S	—	—	D
0.000,1	D	—	—	—

S = survival.
D = death.
— = not done.

Experiments, not shown in the table, demonstrate that autolysate, when injected alone, causes only a mild reaction and is incapable of bringing about infection or death in rabbits.

Another group of rabbits in this series received varying amounts of the same culture of Type III pneumococci, together with heterologous autolysate prepared from organisms of Type I. Under these conditions, as expressed in terms of initial number of infecting organisms, the virulence was also markedly enhanced although not to the same degree as that occurring when the homologous autolysate was used. Whether this slight variation is within the limits of experimental error or represents an actual difference in the action of the two autolysates is not entirely clear, but it is apparent that a marked, if not equal degree of enhancement of virulence can be obtained by the use of an autolysate of Pneumococcus of a type different from that causing the infection.

In a fourth series of animals included in the same experiment the injections were similar except that the autolysate was prepared from a rough strain of Pneumococcus which was entirely avirulent. Here again, results of the same type were obtained. The fact that this particular autolysate was prepared from a culture of R pneumococci seems to indicate that the presence of the soluble specific substance in the autolysate is not essential for enhancing virulence and further, that the factor responsible for this property may be quite independent of those factors upon which virulence ordinarily depends.

These results indicate that pneumococcus autolysates can enhance the virulence of a strain of Pneumococcus which is already somewhat virulent for rabbits, since without the autolysate, 10,000 or more bacterial cells were necessary to bring about fatal disease, whereas when the autolysate was added, less than 10 organisms caused an infection which terminated fatally. Furthermore, these results show that the enhancing factor is not type-specific and that it may be derived equally well from the entirely avirulent, rough strains of pneumococci.

Enhancement of Invasive Properties of a Strain Incapable of Inducing Fatal Infection in Rabbits

The A66 strain of Type III Pneumococcus used in these experiments under ordinary conditions does not bring about death in rabbits when injected intravenously or intradermally in large amounts. On the other hand, it possesses marked virulence for mice, for in these animals amounts as small as 0.000,000,1 cc. of culture invariably prove fatal. This provided the means for determining whether the addition of autolysates, and particularly autolysates from rabbit-virulent strains, might lead to an enhancement of lethal virulence of a strain ordinarily considered to be non-virulent for rabbits.

Preliminary experiments indicated that this was not the case for even when injected together with autolysates prepared from highly virulent pneumococci this strain remained incapable of causing fatal infection. However, the addition of the autolysate did lead to greater invasiveness in the sense that under these conditions fewer organisms were required to produce a widespread area of inflammation. An experiment illustrating this effect is shown in Table II.

TABLE II

Effect of Autolysate on the Infective Power of a Strain of Pneumococcus Which Is Non-Lethal for Rabbits

Culture: Pneumococcus Type III, Strain A66. Does not kill rabbits when given intradermally. Autolysate and culture mixed at time of injection. All injections intradermal.

Amount of broth culture	Materials added to pneumococcus culture	
	0.2 cc. of saline	0.2 per cent of autolysate of virulent Pneumococcus Type III (Strain PH)
cc.		
0.000,000,1		+
0.000,001		++++
0.000,01	+	++++
0.000,1	+	+++
0.001	+++	++++
0.01	+++	++++
0.1	+++	++++

+ = local area of inflammation.
+++ = area of inflammation extending to the ventral midline.
++++ = widespread area of inflammation over midabdominal area.

In no instance did the addition of the autolysate to the non-virulent strain bring about the death of the animal. However, a definite effect was observed in the ability of the autolysate to enhance the spread of the lesion. The results are expressed in terms of the size of the lesion induced by the organisms alone, contrasted with those provoked by the same organisms in the presence of autolysate. It will be noted that when pneumococci were injected alone, an amount of culture as great as 0.1 cc., representing about 100,000,000 bacterial cells, was capable of causing a lesion which spread just to the ventral midline. When a smaller amount, such as 0.000,1 cc. was used, only a circumscribed local area of inflammation was produced. On the other hand, when mixed with autolysate an amount of culture as small as 0.000,001 cc. produced a lesion considerably greater in extent than that resulting from the injection of 0.1 cc. of the culture alone.

The results of this experiment show that, while the invasiveness of a non-virulent organism may be enhanced by the addition of the autolysate of a virulent strain of Pneumococcus, the essential virulence of the organism, in the sense of lethal capacity, is not enhanced in any way.

Effect of Autolysates on the Infectivity of R Pneumococci

The intradermal injection of rough strains of pneumococci in relatively large amounts produces only a local area of inflammation. When autolysates of virulent or non-virulent pneumococci are injected together with this living rough culture, the area of involvement is invariably larger, but never of great consequence. There is little elevation of body temperature and the inflammatory reaction at the site of inoculation is transient.

The experimental evidence obtained under these conditions shows that the invasiveness of the non-virulent rough forms of pneumococci is not appreciably altered in the presence of autolysates which possess the property of enhancing the infectivity of potentially virulent strains of "S" pneumococci.

DISCUSSION

The results of this study show that pneumococcus autolysates have the property of enhancing the potential virulence which a particular strain of pneumococcus may possess. This enhancement is entirely quantitative since there is no qualitative change in the nature of the virulence possessed by the organism. The autolysates seem merely to permit fewer pneumococci to produce the same results as those ordinarily brought about by a much greater number of microorganisms.

The nature of the active substance in the autolysate is not known. Autolysates active in this respect also produce purpura in mice and when added to freshly drawn rabbit blood have the property of inhibiting coagulation. That the virulence-enhancing effect is not type-specific is shown by the fact that an autolysate from organisms of a heterologous type is almost equally effective. Furthermore, an autolysate prepared from a culture of R pneumococci possessed the same power. The result, therefore, is apparently not dependent upon the presence of specific capsular polysaccharide in the autolysate. From the evidence thus far available, it seems not unlikely that the enhancing effect of the autolysate may be due to the presence of a product of protein degradation.

These experiments do not prove, but they lend some weight to the probability that under natural conditions pneumococcus infection may be promoted by the autolysis which some of the infecting organisms undergo. Thus for the rabbit-virulent Type III Pneumococcus it seems likely that the death and autolysis of the less resistant microorganisms may enhance the invasiveness of the few which survive. The relative ease with which pneumococci undergo autolysis suggests the possibility that this mechanism is more likely to be operative in infection with pneumococci than in infections due to many other species of bacteria.

One argument which might be advanced against this view is that with Type I Pneumococcus, as previously reported (1), a minimal number of organisms suffices to produce a fatal infection. In such an instance, however, the long latent period may represent the time required by a few organisms to multiply while others are undergoing autolysis.

Although the results of the present studies suggest certain similarities in action between autolysates, bacterial aggressins (Bail (3)), and virulin (Rosenow (4)), the evidence indicates that in the case of Pneumococcus, at least, the autolysates serve to enhance a property or function potentially present in a given strain rather than to confer a new property.

SUMMARY

In pneumococcus dermal infections in rabbits, the addition of pneumococcus autolysate to an infective inoculum favors the invasiveness of the particular strain employed, but does not alter the kind of virulence possessed by that strain.

Autolysates exhibiting this enhancing property also induce purpura in mice and inhibit the coagulation of rabbit blood. The relation of these properties to the infectivity of Pneumococcus and the possible rôle of bacterial autolysis in natural infection are discussed.

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STUDIES ON A CERTAIN SPREADING FACTOR EXISTING IN BACTERIA AND ITS SIGNIFICANCE FOR BACTERIAL INVASIVENESS

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Plates 11 to 13

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Much would be gained in the understanding of the mechanism of infection if bacterial virulence could be definitely ascribed to certain chemical constituents of virulent organisms. Work in this direction has been extensively carried on, following the important discovery of Avery and Heidelberger (1) of the specific carbohydrates existing in different types of pneumococcus. Granting, as the finding suggests, that each pathogenic organism has individual biochemical characteristics, it is no less true that each shares with other pathogenic bacteria the property of maintaining itself within or actually invading the host tissue to a greater or less extent. Perhaps, as has frequently been suggested, "invasiveness" of an organism may be dissociated from virulence. The latter condition is more specific but varies with general or local susceptibility or resistance on the part of the host and toxigenicity on the part of the bacteria. The phenomenon of the spread of an infection from the point of inoculation or entry is far from being fully understood.

It has been shown in this laboratory that an extractable factor exists in certain animal tissues, notably the testicle, which has the property of enhancing the invasiveness of certain pathogenic agents. Microorganisms of relatively low grade virulence inoculated with testicle extract will produce lesions comparable with those resulting ordinarily from virulent organisms, while those of more virulent type have their infectivity markedly increased by the addition of this factor. Considerable evidence has been accumulated which indicates that the outstanding property of the enhancing factor is the induction of increased tissue permeability. From this it seems probable that the enhancement of infections depends on this property of enabling the infective agents to spread through a larger area of tissue than ordinarily when the organism is inoculated alone. These observations open up the question of whether the invasiveness of an organism may not depend on the existence in it of some factor which renders the tissue more permeable. Goodner's observation (2) (1931) that the advancing edema occurring with pneumococcus skin infections of the rabbit carried with it the infecting organism suggests that an increased permeability of the tissues is induced by the organism. He has extended this observation in experiments (3) more or less paralleling those reported in the present paper.

The outstanding properties of the factor deriving from tissue are that it enhances the infectivity of all bacteria and viruses so far tested (4), it increases tissue permeability as shown by the spread of injected material (5), and possibly increases cell permeabilities as shown by experiments on red blood cells and sea urchin eggs (6). The factor from one species will enhance infections not only in the same species but in all other species susceptible to the infectious agents. Intravenous injection of testicle extract induces a general increase in permeability of the skin with a correspondingly increased susceptibility of this tissue to infectious agents. The active principle is very stable, is soluble in water, and can be isolated from the bulk of soluble tissue proteins and considerably purified (7). In this purified state it will withstand boiling. Available evidence indicates that it lacks antigenic properties (8).

The present paper is a report on a spreading and enhancing factor derived from bacteria together with a comparison between the amount of the factor present and the invasiveness of the organism. The investigation has been carried out mainly on staphylococci with strains of widely differing virulence.

Material and Methods

The strains of staphylococci used were all recently isolated from pathological lesions, the culture in each case being started from a single colony. The material for testing the spreading or enhancing factor was derived from a 24 hour agar slant culture suspended in 10 cc. of water. This suspension in a sealed tube incubated at 37°C. usually showed complete autolysis in 5 to 10 days. Any surviving bacteria were removed by vigorous centrifugation and filtration through a Berkefeld candle. A simpler method used entirely in the latter part of the study was to extract a 24 hour culture with 10 cc. of water and then remove the bacteria. As these two methods yielded products entirely comparable in their action the material will be referred to in the following text merely as bacterial extract. For testing the invasiveness of the organisms, the bacteria from a 24 hour agar slant culture were suspended in 10 cc. of water.

Each strain of bacteria was tested in the following manner. Rabbits were shaved over both flanks. On one side an intracutaneous inoculation was made of 0.75 cc. of organism, representing 0.5 cc. of the suspension diluted with 0.25 cc. of water. On the other side a similar inoculation was made of 0.5 cc. of an extract or autolysate of the same strain of organisms, to which had been added 0.25 cc. of dilute India ink. A third area received an injection of 0.25 cc. of India ink diluted to 0.75 cc. with water. The size of the lesions produced by the bacteria and the area of spread of the ink alone and of the ink with the bacterial extract were recorded 24 hours later. Using careful measurements as a basis, the area covered was expressed for convenience in square centimeters.

In addition to the above described tests the cultural characteristics of each strain were determined by standard bacteriological methods.

Experiments with Staphylococci

For this study 53 strains of staphylococcus freshly isolated from pathological lesions have been employed.[1]

Correlation between the Spreading Factor Content and the Virulence of the Organism.—On the basis of the size and quality of the lesions produced in rabbit skin, the 53 strains of staphylococcus have been arranged in three groups as follows: There were 11 classed as non-invasive or of slight invasiveness, in that the lesions resulting from inoculation were mild and rarely spread beyond the limits of the bleb produced by the inoculation. These lesions were soon resorbed or resolved by the formation of a small abscess, and the healing was prompt. The second group comprised 22 strains considered as having moderate invasive properties. They produced lesions of mixed congestive and necrotic types, accompanied by edema, with a definite spread to surrounding tissues. There was scab formation with accumulation of underlying pus, and healing was slow. The third group included 22 strains considered highly invasive. The lesions

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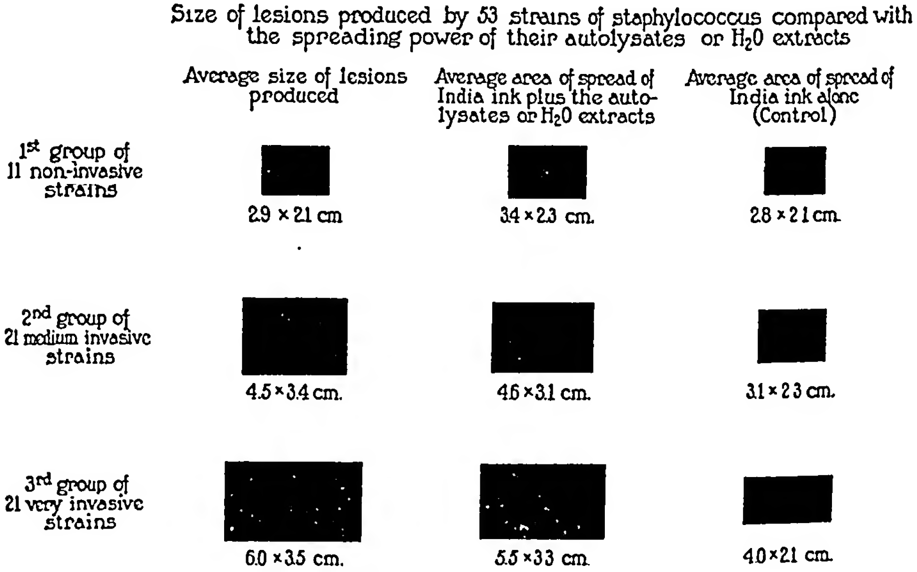
[1] We should like to express our indebtedness to Dr. G. Shwartzman of the Mount Sinai Hospital Laboratories for having supplied us with most of the strains of staphylococcus.

produced were similar to those described above, but were much more extensive, and in some instances resulted in general infection causing the death of the animals. Using the method described above, each strain was tested on an individual rabbit, so that the size of the lesion produced was compared with the amount of spreading factor extractable from the bacteria. Typical examples from each group are shown in Figs. 1 to 6, and the comparative results are illustrated in Text-fig. 1. In the text-figure the area of the lesion and of the spread of the ink are given in square centimeters.



TEXT-FIG. 1

From the photographs and Text-fig. 1 it is evident that a close parallelism exists between the size of the lesions produced and the area of spread of India ink under the influence of the extract of the organism. As with testicle extract, the spread of the product from invasive organisms is easily detected a few moments after injection. While the control material or the extract from non-invasive bacteria remains as an elevated bleb, those formed by the injection of the active material flatten out within a very short time,[2] and within an hour have gen-

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[2] It is noticeable that in the second and third groups of Text-fig. 1 there is a certain spreading in the control injections of India ink alone. The reason for this will be seen later.

erally infiltrated a large area of skin. It is possible to predict with some accuracy the degree of invasiveness of the organism by the area of spreading due to the extract which takes place in the 1st hour after injection. From the above data it seems clear that invasiveness runs parallel with the existence in the bacterial cell of a soluble spreading factor.

Effect of Bacterial Extracts on Infectivity.—The factor in testicle extract which increases tissue permeability seems from all available evidence to be identical with a factor in the same tissue which causes a marked enhancement of infection (5). The enhancement is dependent in all probability on the increased permeability of the tissues to the infecting agent. If this is true the extract of invasive staphylococci should enhance infections. The point has been tested with homologous and heterologous bacteria and with a virus.

Enhancement of Infectivity of Homologous Bacteria.—These experiments deal with the action of extracts from invasive and non-invasive strains of staphylococcus on the infectivity of other strains of the same organisms possessing varying degrees of invasiveness.

The preparation of material was carried out as described above. 0.5 cc. of the bacterial suspension was mixed with 0.5 cc. of the extract. This was injected intradermally in the flank of a rabbit and in the skin of the other side a similar amount of the bacterial suspension diluted with water was injected. Thus the experimental and control materials for each strain were tested on an individual rabbit. The sizes of the lesions were measured 24 hours later and their areas recorded in terms of square centimeters. The results are recorded in Table I.

Enhancement of Heterologous Bacterial Infections.—This group of experiments was similar to that above, but the extracts of invasive staphylococci were tested on other types of organisms including *B. dysenteriae* Flexner, streptococcus, proteus, and *E. typhi*. The results are recorded in Table II.

Enhancement of Virus Infection.—A strain of neurovaccine virus was used as the test material.

To a standard suspension extracts of invasive strains of staphylococcus were added, while the controls consisted of an equal amount of the virus diluted with water. The sizes of the lesions produced were recorded 5 days after the inoculation. For the results, see Table III.

TABLE I

*Effect of Autolysates or Water Extracts from Invasive and Non-Invasive Strains of Staphylococcus on the Lesions Produced by Invasive and Non-Invasive Strains of the Same Bacteria**

Source of bacterial suspension		Lesions produced by 0.5 cc. of bacterial suspension plus 0.5 cc. of H_2O	Lesions produced by 0.5 cc. of bacterial suspension plus 0.5 cc. of autolysate or extract	Source of autolysate or extract	
		sq. cm.	*sq. cm.*		
Non-invasive	Strain 1	8.1	18.5	Strain 13	Invasive
	" 1	8.1	21.2	" 5	
	" 2	8.4	10.2	" 5	
	" 3	4.2	25.5	" 5	
	" 3	4.2	16.0	" 5	
	" 4	6.2	31.2	" 5	
	" 1	6.8	11.6	" 5	
	" 4	5.1	16.4	" 5	
	" 1	7.2	20.2	" 14	
	" 1	8.1	12.2	" 15	Medium invasive
	" 1	8.1	12.2	" 15	
	" 4	6.8	14.4	" 15	
	" 4	6.8	16.0	" 15	
Invasive	Strain 5	21.2	44.9	Strain 14	Invasive
	" 5	19.4	34.8	" 14	
	" 6	21.2	39.1	" 14	
	" 7	15.6	15.6	" 7	
	" 8	16.0	16.0	Strain 15. Medium invasive	
	" 9	16.0	16.0	Strain 1. Non-invasive	
	" 9	16.0	16.0	" "	
	" 9	16.0	16.8	Strain 4. Non-invasive	
	" 5	29.1	29.1	Strain 15. Slightly invasive	
	" 5	29.1	29.1	Strain 1. Non-invasive	
	" 5	29.1	27.0	" "	
	" 5	29.1	26.0	Strain 4. Non-invasive	
Non-invasive	Strain 10	8.4	7.0	Strain 2. Non-invasive	
	" 11	7.0	7.6	" "	
	" 12	6.8	7.0	" "	

* The sizes of the lesions were recorded 24 hours after the injections.

The results of the experiments demonstrate clearly that the extracts of invasive strains of staphylococcus enhance the infectivity of both invasive and non-invasive strains of the same organism. The degree of augmentation seems to bear a definite relationship to the invasiveness of the organism furnishing the extract, the extracts from non-invasive strains being entirely inactive. The enhancement of heterologous bacteria and of a virus infection by the product of invasive strains is also clearly demonstrated. As in the case of the factor present in testicle extract, there is a definite correlation between the enhancement of infections and the spreading produced by the factor.

TABLE II

*Effect of Autolysates from Invasive Staphylococcus on the Lesions Produced by Heterologous Bacteria**

Bacterial species	Lesions produced by 0.5 cc. of bacterial suspension plus 0.5 cc. of H_2O	Lesions produced by 0.5 cc. of bacterial suspension plus autolysate	Source of autolysate
	sq. cm.	*sq. cm.*	
B. dysenteriae Flexner	3.8	16.6	Strain 50
" " "	3.8	7.8	" 56
Streptococcus Rosenow	6.2	12.9	" 50
" "	6.2	14.1	" 56
Streptococcus	9.0	32.3	" 45
Proteus X 19	20.2	32.3	" 50
E. typhi	4.5	26.0	" 50

* The sizes of the lesions were recorded 24 hours after the injections.

TABLE III

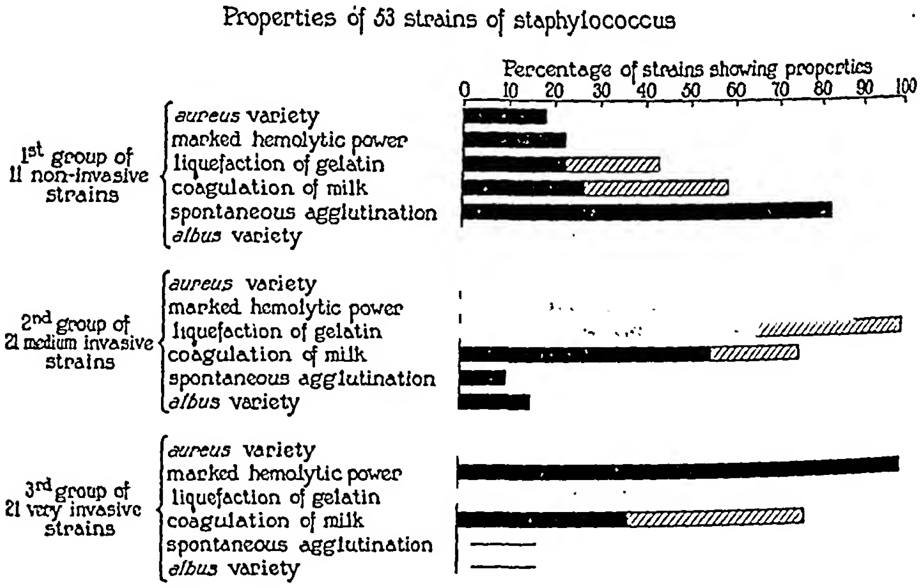
*Effect of Autolysates from an Invasive Staphylococcus on the Lesions Produced by the Neuro Strain of Vaccine Virus**

Dilution of virus	Lesions produced by 0.5 cc. of virus dilution plus 0.5 cc. of H_2O	Lesions produced by 0.5 cc. of virus dilution plus 0.5 cc. of autolysate	Source of autolysate
	sq. cm.	*sq. cm.*	
1:10	23.5	52.6	Strain 50
1:50	15.2	46.9	" 50
1:100	13.1	34.2	" 50
1:500	15.2	26.0	" 56

* The sizes of the lesions were recorded 5 days after the injections.

Correlation between Spreading Value and Cultural Characteristics of Staphylococci.—The cultural characteristics of the 53 strains of staphylococcus used in the foregoing study have been determined in order to see if these exhibited any relationship to the amount of spreading factor.

Standard bacteriological methods have been employed and the percentage of the strains in each group showing particular characteristics has been determined. The results of this investigation are given in Text-fig. 2.



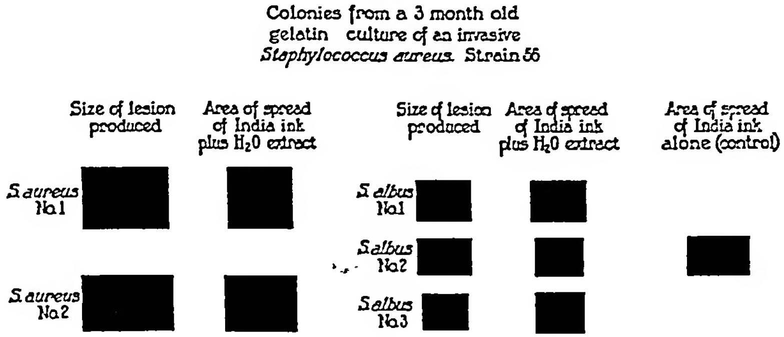
TEXT-FIG. 2

The indications are that the presence of the spreading factor in these bacteria can be correlated in general with the characteristics considered typical for *Staphylococcus aureus*. Deviations from this type are regularly associated with a low content or absence of the spreading factor. This corresponds with the known association of virulence with the typical cultural characteristics for this type of organism.

Conditions Determining Variation in the Amount of Spreading Factor Associated with the Microorganisms.—It has seemed of interest to determine whether conditions which influence the virulence of a particular strain of organism will influence the content of spreading factor.

It is known that virulent strains of staphylococci undergo spontaneous dissociation in old cultures (9). These variants show alterations in color from *aureus* to *albus*, in texture from smooth to rough, and in cohesion from non-viscid to viscid. In addition to the investigation of variants obtained attempts have been made to alter the virulent strains by repeated subcultures and by animal passages under varying conditions.

Experiments with Variants.—Variants were secured from liquefied gelatin cultures of 10 *aureus* strains of staphylococcus. A few colonies of each of the variants were subcultured and tested in rabbits for their infectivity and the spreading property of their extracts. In addition the more important cultural characteristics were determined. The typical R variants, which were white and viscid, with rough colonies, were found to be non-invasive and their extracts showed no spreading factor. In their cultural characteristics, it was found that a change from the original culture had taken place in one or another of the typical reactions; for example, coagulation of milk, liquefaction of gelatin, or hemolytic power. There were several instances of freshly obtained white variants which retained the invasiveness of the original strain, and their extracts contained the spreading factor. These strains were non-viscid, smooth in texture, and had the typical cul-

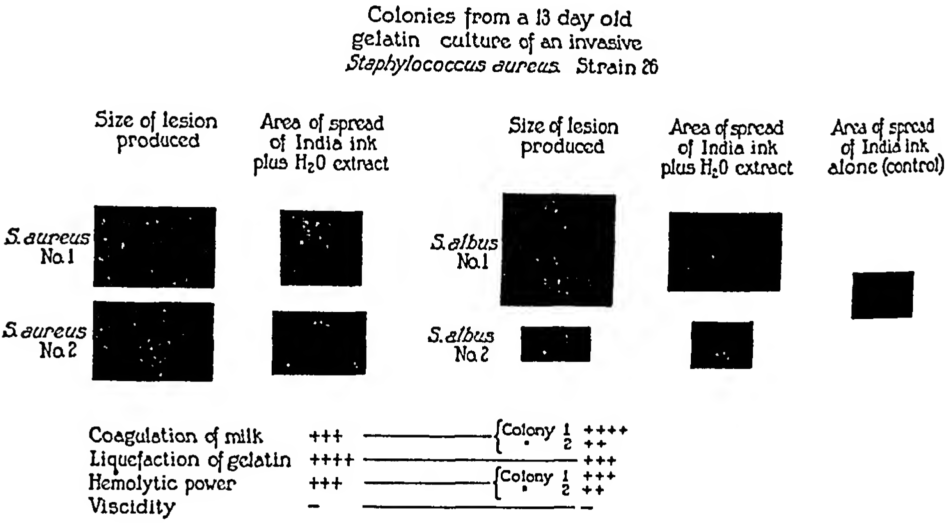


Coagulation of milk	++++	++++
Liquefaction of gelatin	++++	–
Hemolytic power	++++	+
Viscidity	–	++++

TEXT-FIG. 3

tural characteristics of the original organism. Most of these reverted to the *aureus* type in subcultures. Data on the invasiveness, spreading property, and the cultural characteristics of typical examples from amongst the many variants studied are presented in Text-figs. 3 and 4.

The tests have brought out the fact that variants secured from invasive strains of staphylococcus show with loss of invasiveness a loss in the spreading factor as well. When invasiveness is not lost the spreading factor persists.


Colonies from a 13 day old gelatin culture of an invasive *Staphylococcus aureus*. Strain 26
Size of lesion produced
Area of spread of India ink plus H_2O extract
Size of lesion produced
Area of spread of India ink plus H_2O extract
Area of spread of India ink alone (control)
S. aureus No. 1
S. aureus No. 2
S. albus No. 1
S. albus No. 2
Coagulation of milk +++ Colony 1 ++++ " 2 ++
Liquefaction of gelatin ++++ +++
Hemolytic power +++ Colony 1 +++ " 2 ++
Viscidity - -

TEXT-FIG. 4

Spreading Factor in Invasive Strains Repeatedly Subcultured.—2 invasive strains were given 20 passages in 35 days in both agar and broth. At the end of this time animal tests showed that the invasiveness and the spreading property of the autolysates of the organisms exhibited no variation from the properties of the original strains. On the other hand, 1 of the invasive strains subcultured, which had been carried through 30 passages in agar and broth containing 25 per cent glucose, did become modified. The pigment was lost and did not reappear even after 10 passages in plain agar. Invasiveness for the rabbit skin was decreased and paralleling this there was an equivalent decrease in the spreading power of the bacterial extracts.

Effect of Animal Passage on Invasiveness and Spreading Factor.—In attempts to increase invasiveness a non-invasive strain of staphylococcus was submitted to passage by inoculation into the skin, peritoneal cavity, and testicle of rabbits or guinea pigs. The organism was injected alone, and also plus the autolysate of an invasive strain, with its antiserum and with the autolysate of an invasive strain respectively. The strains recovered after these passages, tested for invasiveness and yield of spreading factor, proved to be unchanged in both particulars from the original strain. In further attempts to alter the organisms they were inoculated into animals partially immunized to the same strain; they were carried *in vitro* through broth containing autolysates from invasive strains; and in broth with the corresponding antisera, or both with antiserum and autolysate. None of these manipulations changed either the invasiveness or the amount of spreading factor.

We may conclude from the foregoing experiments that in a variety of manipulations designed to increase or decrease the invasiveness, the parallelism between invasiveness and the spreading factor content remained unchanged irrespective of whether or not these manipulations were successful. Attempts to transform the non-invasive strains to invasive, or to increase the spreading factor by methods successful with the pneumococcus group (10) failed of success with the staphylococci.[3]

Generalized Action of the Bacterial Spreading Factor.—The ability of the invasive staphylococcus cell to elaborate the spreading factor *in vitro* suggests that in all probability it does so *in vivo*. That this is true is suggested by the results of early experiments, shown in Text-fig. 1. India ink injected as control spread further in animals inoculated with an invasive organism at a distance from the point of injection of the ink, than in the animals inoculated with the non-invasive strain. The following experiments constitute a test for a generalized effect of the spreading factor.

Effect of Intravenous Injection of the Spreading Factor.—Preliminary tests showed that the spreading factor present in the bacterial extracts could be easily demonstrated in the serum of a rabbit 5

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[3] Nevertheless Pinner and Voldrich (11) have recently reported that, after many attempts, they succeeded in obtaining virulent *aureus* varieties by growing rough *albus* varieties in their corresponding antisera.

minutes after intravenous injection of such extract. After an hour it was still present, somewhat diminished, but was barely detectable after 3 hours. This rapid disappearance from the blood suggested an elimination, a neutralization, or possibly a fixation by the tissues. The latter possibility was tested by determining the effect of intravenous injection of the factor on the spread of India ink injected into the skin.

5 rabbits were injected intracutaneously in 3 areas each, with 0.75 cc. of diluted India ink, and immediately afterwards 7 to 22 cc. of the bacterial extract was given intravenously. 2 control animals were similarly injected in the skin, but water was substituted for the bacterial extract in the intravenous injections of other control animals. In the control animals the spreading of ink in the skin was insignificant, and at the end of 24 hours covered an area of 5 to 6 sq. cm. On the other hand, the spread of ink in the rabbits injected intravenously with the bacterial extract proceeded very rapidly, so that by the end of an hour the area was considerably larger than in the controls. After 24 hours it covered from 10 to 20 sq. cm. of skin.

Passage of the Factor into the Blood from Local Lesions.—In order to determine whether the spreading factor could be detected in the blood of animals having an infection with the invasive type of organism, animals were inoculated in the testicle and their blood tested at intervals for the factor.

Rabbits were inoculated intratesticularly as follows: 3 with 1/5 of a 24 hour culture of an invasive staphylococcus strain; 1 with 1/10 of the same culture; and 1 with 1/5 of a culture of a non-invasive strain. Samples of blood were withdrawn from each animal before inoculation and at intervals thereafter, and the serum was tested for spreading factor in the usual way.

The 3 rabbits inoculated with 1/5 of the invasive cultures died within the first 24 hours. The sera collected showed a definite but only moderate amount of spreading factor by the usual tests, and the development was parallel with the progress of the local lesion and of the septicemia. The animal which received 1/10 of a culture showed a slower progress of the disease and provided a greater opportunity for studying the process. The presence of the factor was demonstrated, by the usual skin injections, in the blood 3 hours after inoculation and continued throughout the life of the animal. The details of these tests are given in Table IV. The rabbit receiving an inoculation of the non-invasive staphylococcus showed a temporary induration locally, with a brief elevation of temperature, but at no time could the spreading factor be detected in the serum.

The foregoing experiments indicate that the spreading factor injected intravenously has a general effect on the permeability of the skin and that the factor elaborated by the bacteria can be demonstrated in the blood of animals injected with an invasive strain of staphylococcus.

TABLE IV

*Passage of the Spreading Factor into the Blood of a Rabbit from a Testicle Lesion Produced by an Invasive Strain of Staphylococcus**

Sample of serum injected	Area of spread of serum plus India ink	Rectal temperature	Remarks
	sq. cm.	*°F.*	
Before injection	7.2	99.8	
3 hrs. after injection	20.2	101.0	Definite induration of testicle
6 " " "	21.1	101.8	More induration
24 " " "	12.9	100.1	Acute orchitis. Scrotal necrosis
29 " " "	16.0	101.0	Animal very sick
48 " " "	10.8	100.6	Animal worse
60 " " "	12.2	100.1	" "
72 " " "	10.8	101.0	Animal dying. Blood culture +++
96 " " "	11.5	100.6	Animal died
Control. Water plus India ink	6.7		

* Sizes of spreads measured 24 hours after the injections.

Spreading Factor in Other Organisms

Although no extensive study has been made of the spreading and enhancing factor in bacteria other than the staphylococcus, it has seemed important to get some idea of whether the principles established are operative in other bacteria.

*Infective and Spreading Power of 14 Strains of Streptococcus**

Strain used	Origin and virulence or toxicity of the strains	Area of lesion produced by bacterial suspension	Area of spread produced by water extracts	Area of spread produced by India ink alone (control)	Area of lesion produced by whole broth culture	Area of spread produced by broth filtrates	Area of spread produced by India ink plus plain broth (control)
		sq. cm.	*sq. cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*
E-1	Erysipelas, no data on virulence	17.6	16.0	6.7	19.3	14.4	8.5
E-3	" " " " "	16.0	17.6	4.0	25.0	27.0	7.8
090/4/8 hemolytic	Bovine, very virulent† for rabbits and mice	71.0	19.1	5.6			
090/4/9 non-hemolytic‡	Bovine, very virulent for mice and probably for rabbits	53.2	10.6	5.2			
090-R-3054*	Bovine, non-virulent for mice and probably for rabbits	37.2	25.6	6.5	13.6	13.6	11.5
S/43/75§	Measles, very virulent for mice, medium for rabbits	14.8	7.5	6.0	11.7	7.2	3.2
S/43-M.A.	Measles, non-virulent for mice, medium for rabbits	14.0	12.6	4.5	6.2	6.7	3.2
S/43 glossy§	" " " " " " "	6.1	8.4	5.6			
C-203	Scarlatinous, very virulent for mice, not tried on rabbits				4.0	9.0	6.2
K-152	Rheumatic, no data on virulence	7.8	10.0	6.2			
St. A	From normal throat, probably non-virulent	7.2	11.6	7.2			
St. B	Pyogenic, probably non-virulent	5.8	4.8	4.8			
St. C	Old stock strain, non-virulent	7.2	7.2	4.0	9.0	11.6	10.2
Rosenow streptococcus		6.2	8.3	5.0	7.0	10.1	10.1

* The sizes of the lesions were recorded 24 hours after the injections.

† By a virulent strain is understood a strain which kills the mouse when injected intraperitoneally at least in a dilution of 10^{-6} cc. and kills the rabbit when injected intravenously in the amount of 0.1 to 2 cc. of a 16 hour broth culture.

‡ Strain 090-R-3054 was derived from Strain 090/4/8 hemolytic by growing it in immune serum. It has very little specific carbohydrate. Strain 090/4/9 non-hemolytic came from the same culture as No. 090/4/8 hemolytic. Strains 090/4/8 and 090/4/9 have the specific carbohydrate.

§ Strain S/43/75 was derived from Strain S/43-M.A. after 75 passages through the mouse. It contains M substance. Before the passages the strain gave such superficial lesions in the rabbit skin that after 48 hours they were practically gone. After 75 passages the lesions on the 2nd day, although not larger in size, were more raised and conspicuous and took a longer time to heal. Strain S/43 glossy is derived from the same Strain S/43-M.A. and is almost devoid of type-specific substance.

Correlation between Spreading Value and Invasiveness of Streptococcus.—

For this group of tests 14 different strains have been utilized.[4] The lesions produced in the skin of rabbits were similar to those described by Rivers (12). The technique employed was essentially the same as that used in the study of the staphylococcus. The bacteria, grown on plasma or blood agar, were suspended or extracted in water. For some tests whole broth cultures were used and the extracts in these instances consisted of filtrates of cultures. A description of the strains with the results of the tests is given in Table V.

TABLE VI

*Effect of Extracts of Invasive and Non-Invasive Streptococci on Infectivity**

Source of staphylococcus suspension	Areas of lesions produced by 0.5 cc. of bacterial suspension plus 0.5 cc. of water	Areas of lesions produced by 0.5 cc. of bacterial suspension plus 0.5 cc. of bacterial extract	Source of lysate or extract from streptococcus
	sq. cm.	*sq. cm.*	
Strain 1, non-invasive	6.2	27.6	Strain E-1, invasive
" 1, "	6.2	24.0	" E-1, "
" 1, "	6.2	29.2	" E-1, "
" 1, "	6.2	30.2	" E-1, "
" 5, invasive	16.4	30.2	" E-3, "
" 5, "	16.4	30.2	" E-3, "
" 5, "	16.4	29.2	" E-3, "
" 6, "	21.1	22.1	" A, non-invasive

* The sizes of the lesions were measured 24 hours after the injections.

Analysis of the data in Table V shows a general parallelism between the invasiveness for the skin and the existence of the spreading factor in each strain, a finding similar to that observed with staphylococcus. However, Strain 090/4/8 hemolytic and its derivative show a deviation of a quantitative order from this parallelism. Although these are rich in the spreading factor, the areas of their lesions are much

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[4] Dr. G. Shwartzman supplied us with the 2 erysipelas strains. The others, except the last 4 in Table V, were kindly supplied by Drs. McEwen and Lancefield of the Hospital of The Rockefeller Institute. They also furnished the data concerning their virulence in rabbits and mice. The nomenclature used by these workers to designate their strains has been retained.

TABLE VII

*Infective and Spreading Power of 15 Bacterial Species**

Species of bacteria	Origin and virulence or toxicity of the strain	Area of lesions by bacterial suspension	Areas of spreading by water extracts	Areas of spreading of India ink plus water (control)
		sq. cm.	*sq. cm.*	*sq. cm.*
Meningococcus 1	Recently isolated from fatal case of human encephalitis	7.3	9.3	6.0
" 36†	Very old stock strain. Non-virulent	6.3	7.3	6.0
" 44-D†	Highly toxic for rabbits		6.5	4.0
B. dysenteriae Flexner‡	No data on virulence	9.4	11.9	11.9
" " Shiga	Toxic for rabbits. No data on virulence		12.6	4.0
E. typhi‡	Toxic for rabbits. Virulent for mice and rabbits		6.6	4.0
B. coli 1§	Old stock strain. Non-virulent	14.0	11.9	11.9
" " 2	" " " "	9.0	11.9	11.2
B. coli-like 1	From transplantable tumor. No data on virulence	12.5	8.4	5.6
" " " 2	" " " " " " "	8.4	7.3	5.1
Sarcina-like 1	From contaminated culture. Probably non-virulent	9.0	7.3	5.1
" " 2	" " " " "	7.1	7.3	7.0
" " 3	" " " " "	7.8	7.6	7.8
V. cholerae	Old culture strain. Non-virulent for rabbits and guinea pigs	7.8	7.0	7.8
Rickettsia prowazeki‖	Strain kept in tissue culture. Virulent for guinea pigs and human beings	7.8	8.4	8.4

* The sizes of the lesions and areas of spread were measured 24 hours after the injections.

† Supplied by Dr. G. Rake.

‡ Described by Dr. G. Shwartzman (13).

§ Described by Dr. G. Shwartzman (14).

‖ Supplied and studied by Dr. C. Nigg (15).

larger than the areas of spread of their extracts, and in this respect they differ from the other organisms studied. Another point of interest is that some of the strains considered virulent, judged by their ability to kill mice and rabbits, do not contain more spreading factor, and may even have less than other strains considered less virulent. These are examples of the observation that general virulence depends on other factors besides local invasiveness and the spreading factor.

Enhancement of Infectivity by Extracts of Invasive Streptococci.—The enhancing property of the bacterial extracts which has closely paralleled the spreading factor has been investigated for streptococcus. Extracts of invasive and non-invasive strains have been tested with invasive and non-invasive types of the organism by inoculation into the rabbit skin. The results are recorded in Table VI.

The results are entirely comparable to those obtained in the more extensive study of the staphylococcus and show again the close parallelism between the enhancement of infection and the spreading property of the bacterial extracts.

Experiments with Other Types of Bacteria.—In addition to the strains of staphylococcus and streptococcus, some 14 strains of other bacteria have been investigated, and the size of the lesions produced in the skin compared with the spreading property of their extracts. The source and description of this material, with the results of the tests, are given in Table VII.

Among the group included in Table VII are organisms recognized as virulent for man and laboratory animals, yet without marked local invasive power and with a correspondingly low yield of the spreading factor. This is the same condition of affairs which was found to hold for 3 strains of streptococcus in the group reported above.

DISCUSSION

The experiments reported here seem to justify the conclusion that the invasion of the skin by staphylococcus depends on the presence in the microorganism of a substance which markedly increases the permeability of the tissue, thus rendering invasion more easy. The presence of this factor appears to be the principal difference between the invasive and non-invasive type of the bacterium, for the non-invasive type, when injected with the factor derived from invasive

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EXPLANATION OF PLATES

PLATE 11

FIG. 1. Rabbit 6-85, left side. Lesion produced after 24 hours by the intracutaneous injection of 0.5 cc. of a suspension of a 24 hour culture of an invasive strain of staphylococcus in 10 cc. of water. The contour of the lesion has been outlined in ink. The necrotic character of the lesion is not clearly seen.

FIG. 2. Rabbit 6-85, right side. *A*, spread produced after 24 hours by the intracutaneous injection of 0.5 cc. of autolysate of the same strain plus 0.25 cc. of India ink dilution. *B*, spread produced after 24 hours by 0.5 cc. of water plus 0.25 cc. of India ink (control). Notice some secondary spreading due to the action of the spreading factor from the lesion. The contours of the spreads have been outlined in ink.

PLATE 12

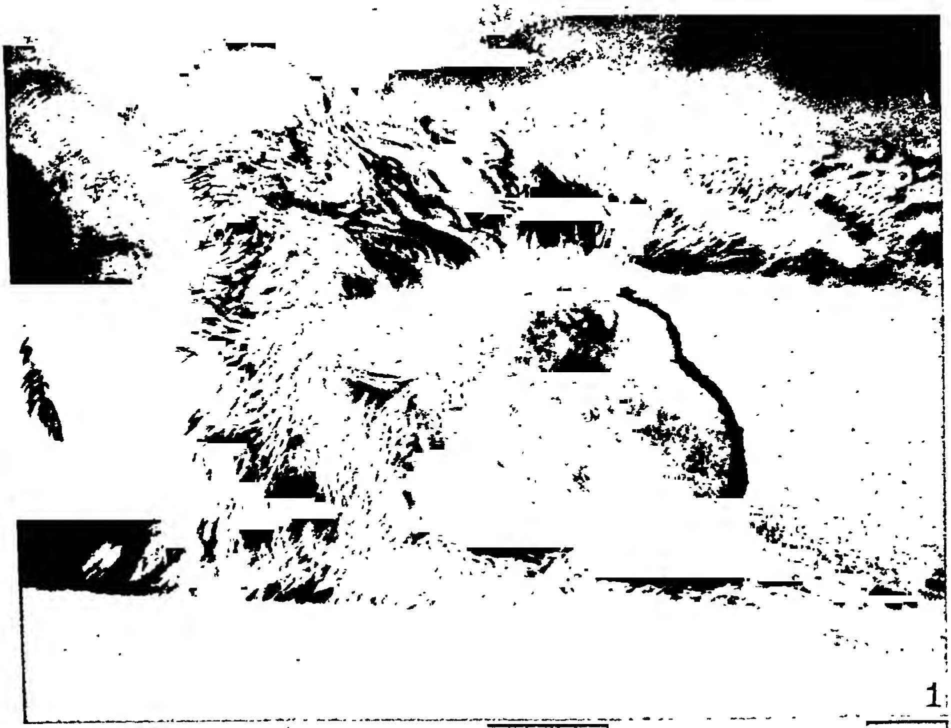
FIG. 3. Rabbit 6-92, left side. *A*, lesion produced after 24 hours by the intracutaneous injection of 0.5 cc. of a suspension of a 24 hour culture of another invasive strain of staphylococcus in 10 cc. of water. The sharp necrotic character of the lesion is clearly seen. *B*, milder lesion produced by 0.5 cc. of a 24 hour broth culture of the same strain. The contours of the lesions have been outlined.

FIG. 4. Rabbit 6-92, right side. *A*, spread produced after 24 hours by the intracutaneous injection of 0.5 cc. of autolysate of the same strain plus 0.25 cc. of India ink dilution. *B*, spread produced by 0.5 cc. of the supernatant fluid of the centrifuged broth culture plus India ink. *C*, spread produced by 0.5 cc. of water plus 0.25 cc. of India ink dilution. Notice the secondary spreading. The contours of the spreads have been outlined in ink.

PLATE 13

FIG. 5. Rabbit 5-00, left side. Lesion produced after 24 hours by the intracutaneous injection of 0.5 cc. of suspension of a 24 hour culture of a non-invasive strain of staphylococcus in 10 cc. of water.

FIG. 6. Rabbit 5-00, right side. *A*, spread produced after 24 hours by the intradermal injection of 0.5 cc. of water extract of the same strain plus 0.25 cc. of India ink dilution. *B*, spread by 0.5 cc. of water plus 0.25 cc. of India ink (control). Notice the absence of secondary spreading.



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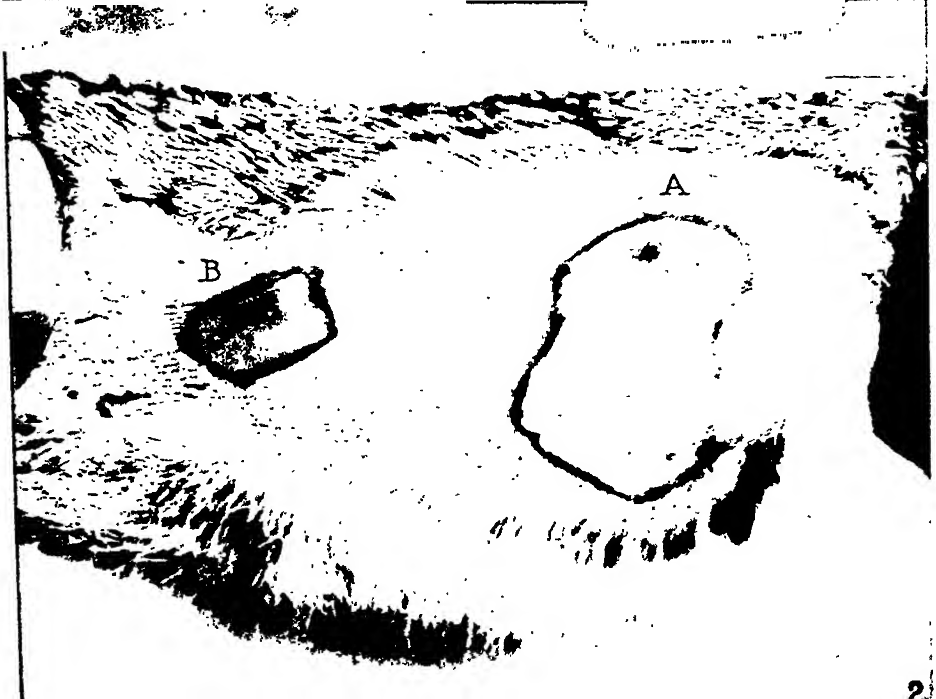

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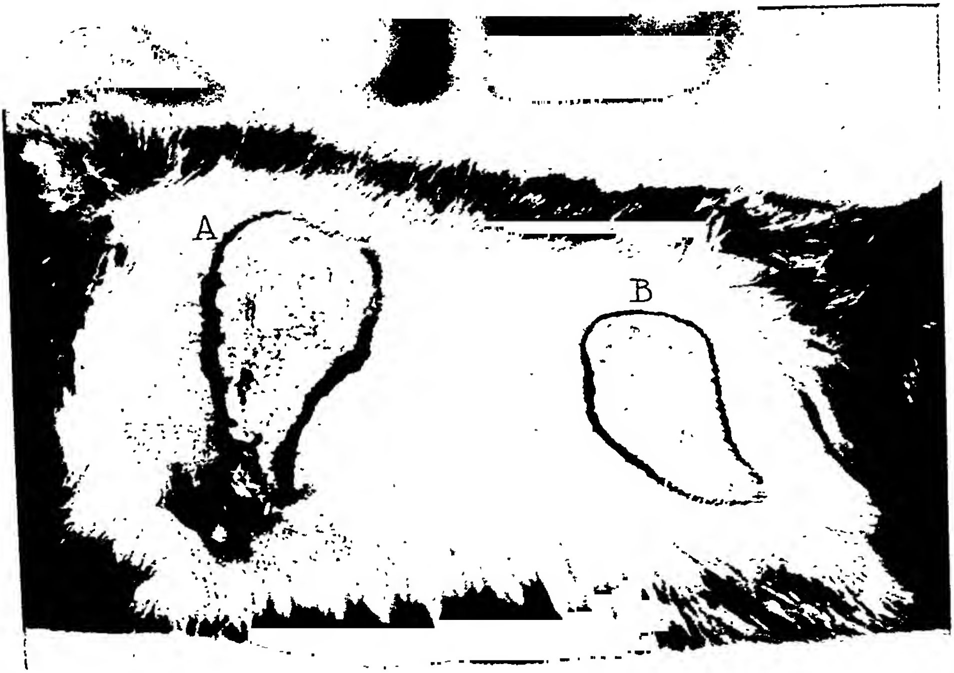

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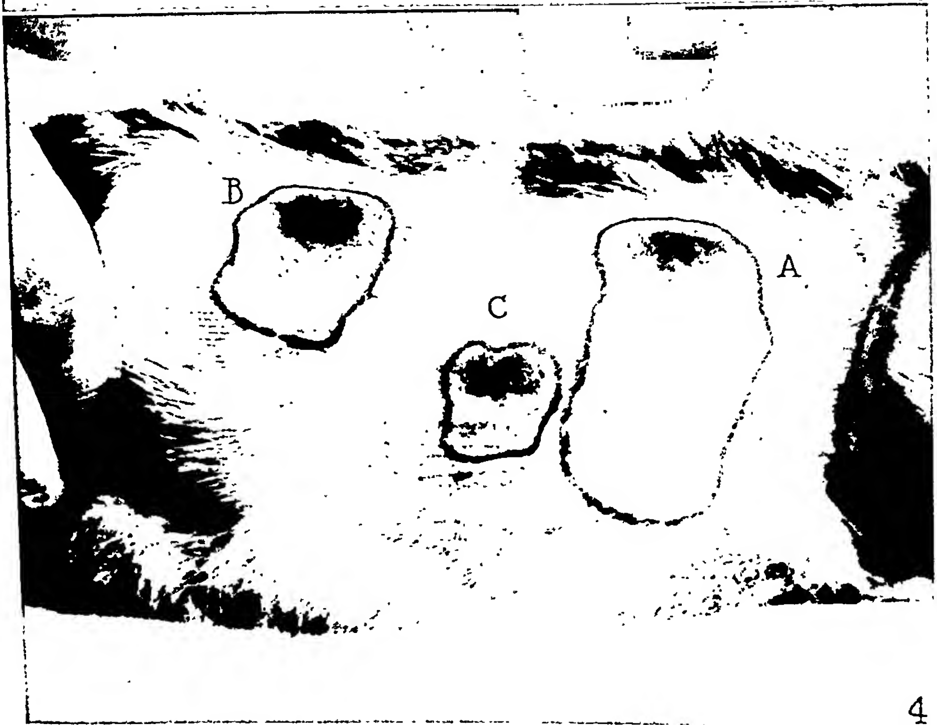

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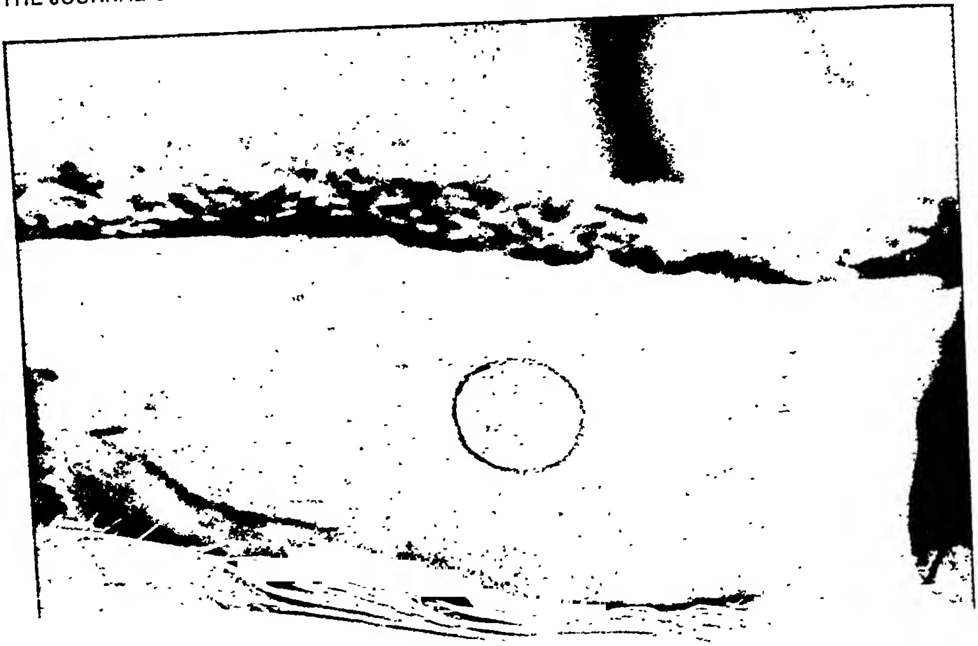

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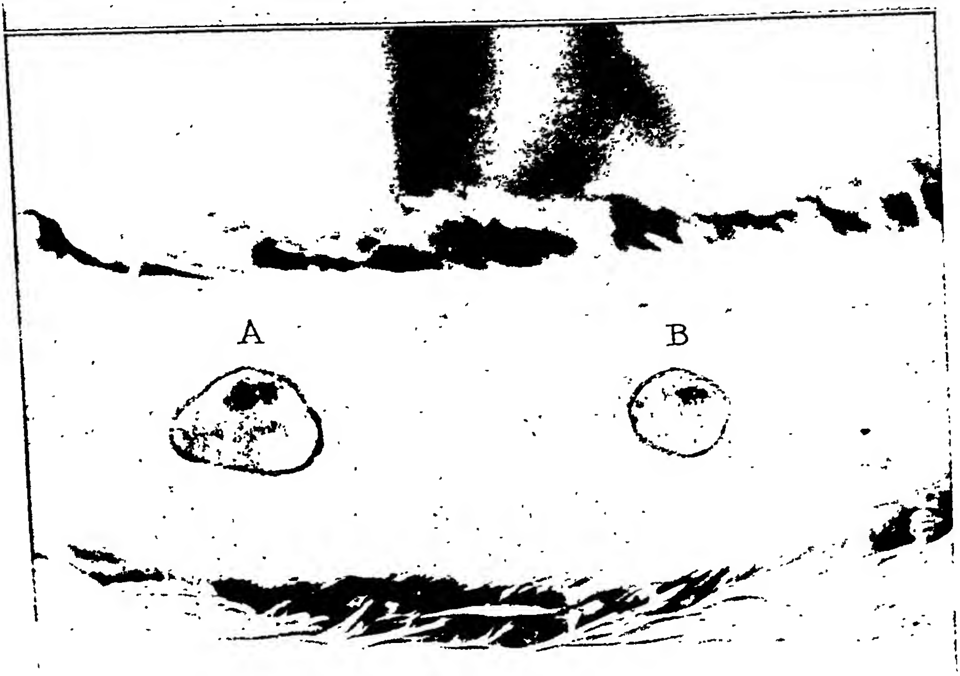

6

(Duran-Reynals: Spreading factor in bacteria)

THE EVALUATION OF ACTIVE RESISTANCE TO PNEUMOCOCCUS INFECTION IN RABBITS

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(Received for publication, May 19, 1933)

The utilization of the dermal pneumococcus infection to determine the active resistance of rabbits has been discussed in an earlier paper (1). Since the publication of that report the method has been applied in the study of an extensive series of animals, so that it has now become possible to evaluate the results obtained by the use of this method.

It is commonly held that if an animal survives following an injection of virulent pneumococci, it is immune, or, conversely, if the animal dies, it is non-immune. Careful study reveals, however, that there are differences in the rate at which animals die, and certainly even greater differences in response to infection among those which survive. An animal's resistance may be of such a character as to bring about recovery, but, nevertheless, the animal may suffer from a severe and protracted infection differing in no considerable respect from one in which the result is fatal. On the other hand, the degree of resistance may be such that the animal develops only a slight localized area of infection and shows no temperature elevation. Thus, while it is possible to stipulate arbitrarily that animals which die are non-immune, and those which survive are immune, it would seem more reasonable to hold that survival and death merely divide the gradient of response to infection into two phases, in each of which considerable differences may be encountered, and that the much abused terms "immune" and "non-immune" describe the two extremes of the scale.

The present paper deals with this gradient of resistance to infection as observed in a large series of rabbits which, previous to infection, had received courses of injection of pneumococci or their derivatives, and in rabbits which had recovered from dermal pneumococcus infections spontaneously or following specific therapeutic measures.

The method of test infection with pneumococci of the various types has been used in a large number of rabbits during the last 8 years. It is manifestly impossible to present in detail all the data which form the basis for the system of evaluation of resistance. The grades of active resistance which will be enumerated have been determined upon after a careful analysis of these data. Even if it were possible to recount all of the experiments, it is doubtful if such data would contribute more than do the illustrative instances presented in this paper. Further evidence will be reported subsequently in connection with studies on the duration of active resistance following immunization.

Methods Used for Test Infections

The general technical procedures are those described in a previous paper on the dermal pneumococcus infection in rabbits (2).

Cultures.—All strains of pneumococci were cultivated and frequently transferred in rabbit blood broth. Repeated rabbit passages were used to maintain the virulence of the organisms. The following strains were used.

Pneumococcus Type I, original Neufeld strain; virulence such that 0.000,000,01 cc. produces a fatal infection in rabbits following intradermal inoculation.

Pneumococcus Type II, Strain D 39; the virulence for rabbits fluctuates slightly, but 0.000,01 cc. given intradermally causes death in a majority of normal rabbits.

Pneumococcus Type III, Strain PH; regularly produces a fatal infection in amounts as small as 0.000,1 cc.

Method of Infective Inoculation.—Each animal to be tested received 0.2 cc. of undiluted 18 hour blood broth culture of Pneumococcus in the skin midway on the flank area.

Observations.—Daily determinations of rectal temperature were made. The appearance of the lesion was carefully followed with regard to the area involved, the amount of edema, and the character of the inflammatory color. In many cases these findings have been supplemented by blood cultures to determine the degree of blood invasion.

The System of Grading of Active Resistance

When the results of a long series of these infective tests are considered, it becomes immediately apparent that in terms of active resistance each animal may be assigned to one of several distinct groups, of which at one extreme is a group including the animals which died promptly, and at the other a group embracing those rabbits which showed greatest resistance to infection. Between these two extremes animals of intermediate resistance fall into groups which may be arranged in a sequential order.

The different degrees or grades of active resistance to infection are listed in Table I, together with the distinguishing criteria.

In Text-fig. 1 are shown temperature charts of rabbits illustrating the characteristic reactions of animals exhibiting the various grades of resistance to dermal infection with Type I Pneumococcus. Entirely comparable results are obtained in animals infected with Pneumococcus Types II and III.

TABLE I

System of Grading of Active Resistance

This system of classification is based on the reactions which follow the intradermal injection in rabbits of 0.2 cc. of an 18 hour blood broth culture of virulent pneumococci.

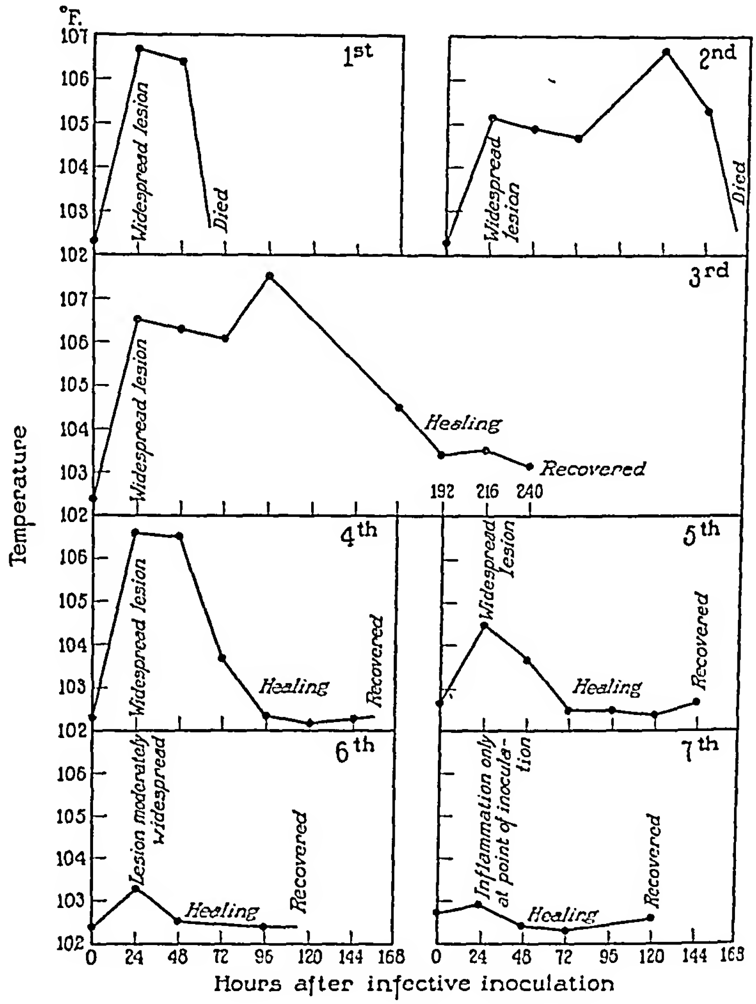
Grade	Reaction after infective inoculation			
	Febrile reaction		Extent of lesion	Ultimate result
	Maximum temperature	Duration		
1	High	Until death	Widespread	Death (within 4 days)
2	"	" "	"	Death (after 4 days)
3	"	5 days or longer	"	Survival
4	"	Less than 5 days	"	"
5	Elevated but less than 105.5°F.	" " 5 "	"	"
6	No significant elevation	—	Moderate to widespread	"
7	No elevation	—	Local	"

The characteristics of the various grades of resistance may be defined as follows:

Grade 1.—This grade is assigned to all animals which develop a typical edematous dermal infection and exhibit a high temperature persisting until death, the latter occurring within 4 days. This arbitrary time period has been selected because it covers the course of the infection in most normal controls.

Grade 2.—This grade is allotted to those rabbits which die after 4 days. Many such cases are encountered, especially with Type III infections. These animals are apparently able to withstand the infection or hold it in check for many hours, only to die as the resistance is gradually overcome.

Grade 3.—Animals which show a typical widespread lesion and a high temperature persisting for 5 days or longer but which eventually survive, are given the rating of Grade 3. In some cases the recovery is abrupt, while in others the febrile reaction is of long duration and only gradually subsides to normal levels.



TEXT-FIG. 1. Temperature charts of rabbits which have been chosen as representative of the various grades of active resistance to pneumococcus dermal infection.

Grade 4.—Those rabbits which develop a very high rectal temperature (105.5°F. and above), but in which fever does not persist as long as 5 days, are given Grade 4. These cases also show a widespread edematous lesion. All animals in this group survive. The shorter febrile course is regarded as a significant feature.

Grade 5.—The grading of 5 is assigned to those animals which show a temperature of transient character ranging up to 105.4°F., 24 hours after infection. The tendency to early termination of the disease process is even more pronounced than in Grade 4.

Grade 6.—This grade is assigned to those animals which develop a lesion of moderate to considerable spread, but which fail to show a significant elevation of body temperature (temperatures below 104.0°F. are regarded as within the normal range). The lack of a marked febrile reaction distinguishes this from Grade 5, and the presence of a spreading lesion sets it apart from Grade 7.

Grade 7.—The rabbits designated by this grade show an entire absence of temperature elevation 24 hours after infection. There is always a slight localized area of inflammation at the point of inoculation, but this shows no tendency to spread.

Two other grades or degrees of resistance may be postulated, one at either extreme of the general scale:

The first additional grade, which may be designated as No. 0, is assigned to those previously infected animals in which the general physiological resistance has been so lowered by severe intoxication, poisons, and other deleterious influences, that the presence of the infecting organisms excites almost no local reaction, and there occurs a rapid, generalized invasion by the bacteria. The body temperature is depressed rather than elevated and the animal quickly dies. This condition is due to extraneous influences and is not of importance in a consideration of the natural gradient of resistance. Examples of this order have been cited in a previous paper (3).

The second additional grade is largely hypothetical, and is designated as No. 8. In a very few instances, in over 500 test infections, no inflammation of any sort occurred at the point of inoculation. These few animals had received many courses of injections with heat-killed pneumococci prior to the test infection, and might be thought of as hyperimmune. Although a rarity, and perhaps an accident, such a state of immunity must be considered as a possibility.

Illustrative Examples of the Application of the System of Grading

In order to make this system of grading of resistance more comprehensible and to demonstrate the method of application, two series of tests for active resistance in rabbits will be cited. Each experiment will be considered as an independent entity which, although undertaken in connection with other work, is used here as an illustrative example.

Experiment 1. Active Resistance to Infection in Rabbits Previously Given Intravenous Injections of Heat-Killed Suspensions of Pneumococcus Types I, II, and III.—Six rabbits were each given identical courses of intravenous injections of a heat-killed bacterial suspension consisting of Types I, II, and III pneumococci in equal proportions. 10 days after the last immunizing injection two of these rabbits were infected intradermally with Type I pneumococci, two with Type II, and two with Type III. Normal controls were likewise infected. The results of these test infections are shown in Table II.

TABLE II

Results of Test Infections in Rabbits Previously Given Intravenous Injections of Heat-Killed Suspensions of Pneumococcus Types I, II, and III

Each of the first six rabbits had received identical immunizing courses of intravenous injections of a vaccine composed of equal numbers of heat-killed pneumococci of Types I, II, and III. Test infections as indicated, 10 days after last injection of bacterial suspension.

Rabbit	Character	Test infection with Pneumococcus Type	Reaction after infective inoculation				Grade assigned
			Febrile reaction		Extent of lesion	End-result	
			Maximum temperature	Duration			
			°F.				
A	Immunized	I	103.4	—	Local	S	7
B	"	I	103.4	—	"	S	7
C	"	II	103.5	—	Widespread	S	6
D	"	II	103.3	—	"	S	6
E	"	III	104.4	1 day	"	S	5
F	"	III	104.1	1 day	"	S	5
G	Normal control	I	?	?	"	D 1	1
H	" "	II	105.1	Until death	"	D 4	1
I	" "	III	108.0	" "	"	D 3	1

S = survival.
D = death, at indicated number of days after infective inoculation.

From the results of this experiment, it will be noted that the animals infected with Type I pneumococci developed only a local lesion and showed no febrile response. The resistance has been given the grade of 7. The rabbits infected with Type II developed a widespread lesion, but did not show a febrile reaction, and the grade of 6 has been assigned. The rabbits infected with Type III also developed a widespread edematous lesion, but in addition developed a fever which persisted for a day. These have been classed as showing resistance of the fifth grade. All normal controls died within 4 days and their resistance has consequently been rated as that of Grade 1.

That these animals possessed a higher resistance against Type I pneumococci than against Type II, and higher resistance to Type II than to Type III pneumococcus infection, is not surprising in view of the fact that the antigenic capacities of these types range in the same order. While there is evidence that these animals were really immune to Type I pneumococcus infection, since there was no spread of the infectious process and no systemic reaction, it is doubtful if the same term can be correctly applied in the other cases, since the infections with Type II and Type III pneumococci gave rise to processes

TABLE III

Active Resistance to Infection in Rabbits Previously Given Intravenous Injections of Pneumococcus Autolysate

Each of the first six rabbits had received identical courses of intravenous injections of bacterial material; three of these being treated with heat-killed Type I pneumococci, and three with an autolysate of an equivalent amount of bacteria. Test infections as indicated were carried out 10 days after last injection of bacterial material.

Rabbit No.	Immunizing courses with	Test infection with Pneumococcus Type	Reaction after infective inoculation				Grade assigned
			Febrile reaction		Extent of lesion	End-result	
			Maximum temperature	Duration			
			°F.				
1	Heat-killed Type I pneumococci	I	102.9	—	Local	S	7
2	" "	I	102.5	—	"	S	7
3	" "	III	106.6	11 days	Widespread	S	3
4	Autolysate of equivalent suspension	I	106.3	Until death	"	D 2	1
5	" "	I	106.4	8 days	"	S	3
6	" "	III	106.3	Until death	"	D 3	1
7	Normal control	I	106.6	" "	"	D 4	1
8	" "	III	106.6	" "	"	D 4	1

S = survival.
D = death, at indicated number of days after infective inoculation.

which were not limited to the point of inoculation, and in the animals infected with Type III Pneumococcus there was a definite systemic reaction.

Experiment 2. Active Resistance in Rabbits Previously Given Intravenous Injections of Pneumococcus Autolysate.—This experiment was designed to determine the degree of active resistance brought about by injections of a heat-killed suspension of Type I pneumococci as compared to that produced by the injection of an autolysate of the same organisms.

A suitable suspension of washed Type I pneumococci was divided into two equal parts. The first portion was immediately heated at 65°C. for 30 minutes, and the second was allowed to autolyze until no formed elements remained and then was heated in a similar manner. Identical courses of intravenous injections of these materials were then given to rabbits, three animals receiving the heat-killed organisms, and three the autolysate. 2 weeks after the last injection two rabbits of each group were infected with Type I pneumococci, and one from each group was infected with Type III. Normal controls were infected with each type. The results of this experiment are shown in Table III.

Those animals which received the intact bacterial cells were immune against Type I infection, as shown by the failure to develop a febrile reaction and the fact that the infective process remained entirely localized. The rabbit infected with Type III survived the infection, but only after a long febrile period of 11 days. On the other hand, the animals which had been given injections of pneumococcus autolysate showed almost no resistance against either Type I or Type III. One rabbit survived infection with Type I, but only after a long febrile disease. The normal controls died promptly.

This experiment particularly emphasizes the failure of the criteria of survival and death as a basis for judgment of active immunity. By the application of the scale of grading it is easily possible to conclude (*a*) that following immunization with heat-killed suspensions of pneumococci the resistance is high and specific, and (*b*) that previous injections of pneumococcus autolysates confer little if any active resistance.

Many similar instances of the inherent fallacies of the survival-death system of evaluating immunity or resistance have been observed. Animals immunized with a given type of pneumococci, if tested at the proper time, show relatively high resistance to infection with the homologous type, and almost invariably survive. If infected with pneumococci of an heterologous type, the degree of resistance is always low, but some of the animals may survive and thus give an impression, if the circumstances are not understood, of non-type-specific active immunity. Similarly, if animals are immunized with rough pneumococci, some may survive test infection with virulent smooth forms, but the degree of resistance, if determined in the manner described, is low in every instance.

The question of the relation of the degree of resistance to the titer of specific antibodies of the serum of the test animal will be discussed at length in a subsequent paper, but it may be stated that these two factors do not necessarily parallel each other.

DISCUSSION

In discussing the gradient of resistance to infection, it may be well to present the evidence now available as to the significance which can be attached to the various degrees of resistance as defined in this paper.

While it is generally assumed that normal rabbits possess little active resistance to pneumococcus infection, we have observed that an occasional normal animal may recover spontaneously from Type I and Type III pneumococcus infections, although only after a typical febrile course (2, 4). These cases would be classed as showing a Grade 3 resistance. A few normal animals react in such a way that their resistance would be rated as Grade 2. By far the greater number (about 98 per cent) show a complete lack of resistance (Grade 1). Thus, normal animals which by commonly accepted definition are non-immune and possess no specific antibodies, may be found in each of the first three groups or grades. The type of reaction illustrated by Grade 3 is also frequently encountered in animals immunized to heterologous types of pneumococci, and to derivatives of pneumococci. These animals show no type-specific antibodies.

Grade 7 represents the highest active resistance demonstrable under these conditions in a majority of instances. The infection fails to spread and there is no systemic reaction. Examples of this degree of resistance are found only among those animals which have been specifically immunized, either actively or passively. This high degree of resistance is invariably associated with relatively high specific antibody titers.

Grade 6 also must represent a high degree of resistance, for, although the lesion spreads and in this respect resembles the less immune animals, there is almost a total absence of systemic reaction.

Grades 4 and 5 present a much more difficult subject for analysis. These grades have some features in common: a widespread edematous lesion and definite febrile reaction. In neither instance does this temperature persist as in Grade 3, a feature of special significance. Grades 4 and 5 are differentiated arbitrarily by the degree of this febrile response. The fever level in rabbits is about 104.0°F., and points over this are regarded as significant. In Group 5 the temperature always exceeds this level, but by definition does not exceed 105.4°F. nor persist longer than 4 days. The mechanism involved in bringing about the abbreviation of these infections is not understood.

Although it is obvious that the virulence and the dosage of the invading organisms play a considerable part in the matter of the establishment of an infectious process, these complicating factors have been eliminated in the present experiments by the use of constant doses of pneumococci of almost constant virulence for the animals employed. It may be pointed out that at least two elements are concerned in these evaluations of active resistance. The first is the element of specific resistance, usually thought of as associated with antibodies, either cellular or circulating. The second factor is that of the capacity of the animal body to react in such a way as to make possible the utilization of these specific elements; this appears to be the underlying basis which conditions the functioning of the influences which find expression in the phenomena of specific resistance.

Aside from the primary purpose of this report, another matter of possible importance may be referred to. It has been shown that in animals possessing varying degrees of active resistance to pneumococcus infection any one of a series of reactions, from the most mild to the most severe, may be encountered. These reactions parallel in many instances the varieties of lobar pneumonia seen in man,—the fulminating type; the cases which die after a long febrile course; those which undergo recovery by crisis or lysis; the abortive type; the cases which have definite pulmonary involvement but little systemic reaction; and other varieties. It is suggested that these degrees of clinical severity of lobar pneumonia may likewise indicate differences in the individual potentialities of active resistance.

The data presented in this paper serve to emphasize that the terms "immunity" and "resistance," so frequently used in a positive or negative sense, can only be considered as relative. The mere fact that an animal survives a test infection leads to a wholly inadequate conception that this animal is immune or resistant. For example, if the commonly used experimental terms were applied to clinical practice, any patient who survives lobar pneumonia, no matter how severe the case, might be said to be actively immune at the beginning of the infection. Obviously, the term active immunity, in the positive sense, must refer to the ability to prevent or at least sharply limit infection, and the term non-immune, in the negative sense, to the total inability to cope with an infectious agent. A majority of cases of natural infection in man fall between these two extremes. It would seem that our understanding of infection and resistance must involve other criteria besides the question of survival and death in bacterial disease.

SUMMARY

The dermal pneumococcus infection has been employed to determine the active resistance of rabbits against infection, and an evaluating scale of the gradient of resistance has been established. The significance of the various degrees of resistance has been discussed, and the possible general inferences of the findings indicated.

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RESISTANCE TO PNEUMOCOCCUS INFECTION IN RABBITS FOLLOWING IMMUNIZING INJECTIONS OF HEAT-KILLED PNEUMOCOCCUS SUSPENSIONS

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Many studies have been published in regard to the antibodies developed in animals following injections of bacterial suspensions, but these have seldom directly concerned the question of the induced resistance against infection. This, to some extent, is due to the difficulties in evaluating the various phenomena associated with experimental infections.

In an earlier paper (1), dealing with the dermal pneumococcus infection in rabbits, certain experiments were reported in which this method of infective inoculation was employed in an effort to determine the active resistance or immunity of these animals against Type I pneumococcus infection after specific conditioning by various means. A system of grading the relative degrees of active resistance against pneumococcus infection has been formulated on the basis of the large number of test infections which have subsequently been carried out (2). From the data presented it was concluded that the commonly accepted criteria for the evaluation of specific active resistance, *i.e.* survival and death, are misleading, for they merely divide the gradient of response to test infection into two phases, in either of which considerable differences may be observed.

The present paper deals with the degree and duration of the active resistance to infection observed in rabbits following the intravenous injection of heat-killed pneumococci. The presence of type-specific agglutinins and protective antibodies in the sera of these rabbits is compared with the degree of active resistance shown by the same animals.

EXPERIMENTAL

The general plan of this project was as follows: A large number of rabbits were given simultaneously a short course of intravenous injections of heat-killed Type I, Type II, and Type III pneumococci in equal proportions. At regular intervals for a year, animals, not previously tested, were infected with each of the three types of pneumococci to determine the degree of active resistance. Just before infection, the animals were bled and the titers of agglutinins and of type-specific protective substances in the serum were determined.

Cultures.—The cultures used for both immunization and infection were as follows:

Pneumococcus Type I (Neufeld strain); virulence such that 0.000,000,01 cc. produces fatal infection in rabbits following intradermal inoculation.

Pneumococcus Type II (Strain D39); the virulence for rabbits fluctuates somewhat, but 0.000,01 cc., given intradermally, causes death in a majority of the animals.

Pneumococcus Type III (Strain PH); regularly produces fatal infection in rabbits in amounts as small as 0.000,1 cc., given intradermally. Regular transfers are made in rabbit blood broth. Frequent rabbit passages are used to maintain the virulence of the organisms.

Preparation of Bacterial Suspensions for Immunization.—Heat-killed pneumococci of each of these strains were suspended in physiological saline in a concentration such that 1 cc. of the suspension represented the bacterial cells contained in 10 cc. of the original broth culture. Equal volumes of suspensions of the three types of pneumococci were then pooled so that the final preparation contained approximately the same number of organisms of each type.

Method of Immunization.—Rabbits were given three intravenous injections at 4 day intervals, each injection consisting of 3 cc. of the pooled bacterial suspension.

Selection of Animals for Testing.—At each test period following immunization a group of from six to nine rabbits, not previously examined, was selected at random. Two to three animals of the selected group were then tested against each of the three types of Pneumococcus, to determine the presence of circulating type-specific antibodies and the degree of active resistance to infection. Normal control rabbits were also infected at each test period with each type of Pneumococcus. In determining the duration of induced active resistance it is obviously possible to test each animal only once, since each test infection, provided the animal survives, may serve to alter the degree of resistance due to the original specific immunization.

Determination of Agglutinins.—The presence of agglutinins was determined by a modified thread reaction (3). To 1 cc. of a dilution of rabbit serum was added 0.2 cc. of an actively growing broth culture of Pneumococcus of the type in question.

The tubes were then incubated for 2 hours in the water bath at 37°C., placed in the ice box overnight, and the reactions read the next morning. Agglutinins were recorded as present in the serum only when the reactions were positive in a serum dilution of at least 1–10.

Determination of Protective Antibodies.—The presence of protective antibodies in the blood of the immunized rabbits was demonstrated in the usual manner by determining the largest amount of culture against which 0.2 cc. of serum would protect white mice, the immune serum and culture being injected intraperitoneally at the same time. The virulence of these strains of pneumococci for mice was such that 0.000,000,1 cc. of a broth culture, given intraperitoneally invariably induced a fatal infection. The protective titer is recorded as negative if 0.2 cc. of the serum failed to protect against 0.000,001 cc. of culture.

Method of Determining Active Resistance.—Each rabbit to be tested against a particular type of Pneumococcus was given an intradermal inoculation of 0.2 cc. of an 18 hour blood broth culture of that type. The general details of this method have been previously described. Daily determinations were made of the body temperature. The appearance of the dermal lesion was carefully followed with regard to the area involved, the amount of edema, and the character of the inflammatory color, purpura, etc. These observations were frequently supplemented by quantitative blood cultures to determine the degree of blood invasion.

Method of Evaluating the Active Resistance.—The method of evaluating the results of the test infections in terms of degrees of active resistance has been described in detail in the previous paper (2). The various grades have been arbitrarily established on the basis of hundreds of test infections and certain general rules permit an effective classification of any similar test infection.

For the purposes of this presentation, each animal will be regarded as immunized only against that type of Pneumococcus with which it is subsequently tested. The possible effect of a crossing or overlapping resistance will be considered later.

Induced Active Resistance against Type I Pneumococcus Infection

Although it is well recognized that Type I Pneumococcus possesses a relatively greater antigenic capacity than do the other types of pneumococci, it is also true that it is the more highly virulent when injected intradermally in rabbits. These two qualities might be expected to be somewhat mutually compensatory as far as the determinations of resistance in immunized animals are concerned. The use of this type of Pneumococcus offers an excellent experimental condition since even such a moderate immunizing procedure as that employed might be expected to give rise to the formation of antibodies and thus to afford an opportunity to determine any correlation existing between the presence of these substances in the blood and the degree of active resistance to test infection.

TABLE I

Degree and Duration of Active Resistance to Type I Pneumococcus Infection in Rabbits Following Intravenous Injections of Heat-Killed Suspensions of Pneumococci (Types I, II, and III)

Test interval	Rabbit No.	Serum antibodies		Test infection (Type I Pneumococcus)				
		Agglutinin titer	Protective titer	Febrile reaction		Extent of lesion	End-result	Grade of resistance
				Maximum	Duration			
days			*cc.*	*°F.*				
10	1	1–20	0.1	103.4	—	Localized	S	7
	2	1–50	0.1	103.4	—	“	S	7
mos.								
1	1	1–10	0.1	103.0	—	“	S	7
	2	1–40	0.1	104.4	1 day	Moderate spread	S	5
	3	1–10	0.1	103.6	—	“ “	S	6
2	1	—	0.001	105.2	3 days	Widespread	S	5
	2	—	0.001	106.5	2 days	“	S	4
	3	—	0.000,1	106.9	8 days	“	S	3
3	1	—	0.01	106.6	6 days	“	S	3
	2	—	0.1	103.6	—	“	S	6
	3	1–10	0.1	104.6	1 day	“	S	5
4	1	—	0.01	104.2	1 day	“	S	5
	2	—	0.001	105.8	2 days	“	S	4
	3	—	0.01	104.9	1 day	“	S	5
5	1	—	0.001	105.6	3 days	“	S	4
	2	—	0.01	104.4	3 days	“	S	5
	3	—	0.001	106.6	Until death	“	D 3	1
6	1	—	0.001	106.1	9 days	“	S	3
	2	—	0.001	106.2	2 days	“	S	4
	3	—	0.01	104.5	1 day	“	S	5
7	1	—	0.000,1	105.1	1 day	“	S	5
	2	—	0.000,1	106.2	Until death	“	D 3	1
	3	—	0.001	106.5	“ “	“	D 5	2

S = survival.

D = death, at indicated number of days after infective inoculation.

The agglutinin titer is expressed in the highest dilution in which agglutination occurred with the type of pneumococcus homologous to that used in the test infection. A minus sign indicates that no agglutinins were demonstrable.

The protective titer is expressed in the largest amount of culture against which 0.2 cc. of the serum protected mice from infection with the type of Pneumococcus homologous to that used in the test infection.

TABLE I—*Concluded*

Test interval	Rabbit No.	Serum antibodies		Test infection (Type I Pneumococcus)				
		Agglutinin titer	Protective titer	Febrile reaction		Extent of lesion	End-result	Grade of resistance
				Maximum	Duration			
mos.			*cc.*	*°F.*				
8	1	—	0.000,1	106.8	Until death	Widespread	D 4	2
	2	—	0.1	105.9	" "	"	D 3	1
	3	—	0.000,1	107.5	7 days	"	S	3
9	1	—	0.001	105.0	2 days	"	S	5
	2	—	0.000,1	106.7	Until death	"	D 3	1
	3	—	0.000,1	106.0	" "	"	D 3	1
10½	1	—	0.000,001	106.9	5 days	"	S	3
	2	—	0.000,001	105.1	Until death	"	D 3	1
12	1	—	0.000,001	106.5	" "	"	D 12	2
	2	—	0.000,1	104.4	" "	"	D 4	1
	3	—	0.000,001	106.6	5 days	"	S	3

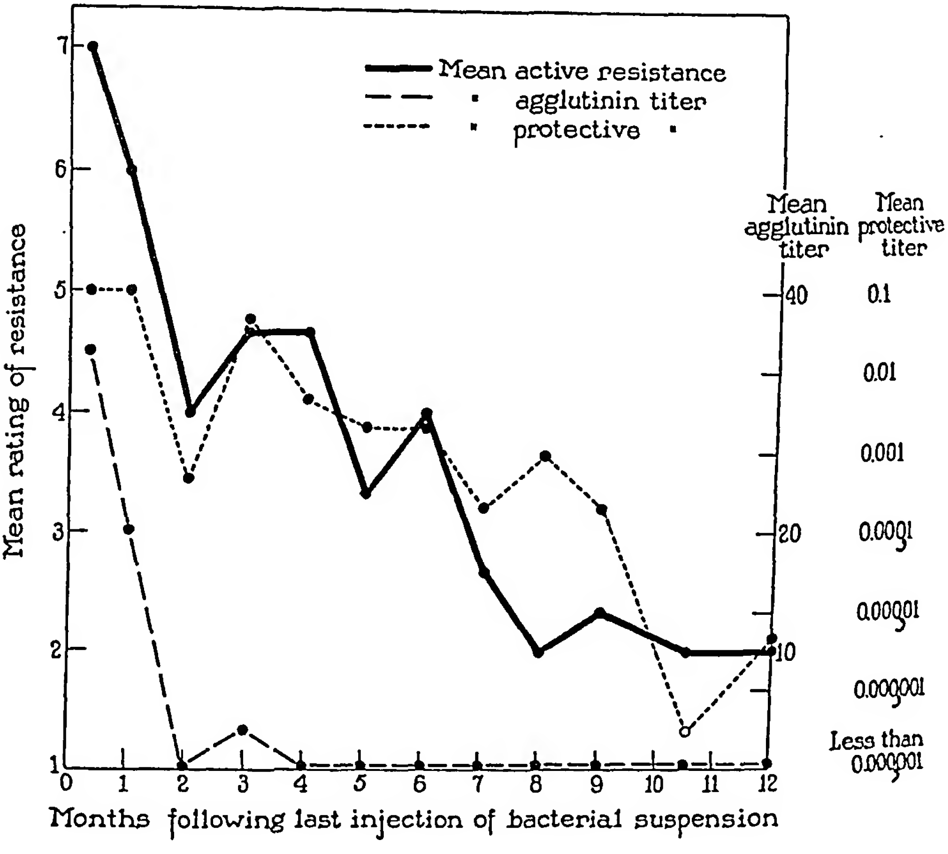
The data regarding the resistance of these artificially immunized animals against Type I pneumococcus infection at various periods during the year following the immunization process are summarized in Table I, together with data on the antibodies demonstrable in the sera of these rabbits at the time of infection.

For the purpose of graphic presentation the mean values of each of the three determinations are plotted in Text-fig. 1. It is obvious that too few cases are involved to give any considerable significance to these average figures but they do serve to indicate the general trends.

Agglutinins.—From the results shown in Table I, it will be noted that agglutinins were found in the sera of these rabbits up to and including the first month in a titer of 1–10 or better, but after that time they were demonstrable in only a single instance. This finding is entirely consistent with the results previously reported by one of us (5).

Protective Substances.—From the data concerning the titer of the protective antibodies it will be noted that there is a considerable individual variation. The passive protection was high in all cases during the 1st month. Thereafter the protective substances were uniformly present but in widely varying degree. If one calculates the average protective titer at each test period there is a slight but definite tendency to decrease during the 12 month period. In general, these findings are consistent with the results in previously reported experiments in which it was shown that a detectable titer of protective antibodies may persist for several years in the serum of rabbits immunized with Type I pneumococci (5).

Active Resistance.—High active resistance was shown in animals tested 10 days after the completion of the immunizing course. Again at the 30 day period the responses to infection were indicative of a rather effective degree of resistance. During the next 4 months the results were irregular but the general level was much lower. Beginning at the 7th month the level of resistance again dropped and then remained low although a few individual animals still showed a moderate resistance. In spite of a considerable individual variation it is



TEXT-FIG. 1. Charts of the degree and duration of active resistance to Type I pneumococcus infection and of specific serum antibodies in rabbits following intravenous injections of heat-killed suspensions of pneumococci (Types I, II, and III). The points represent the means of the various determinations at each test period.

TABLE II

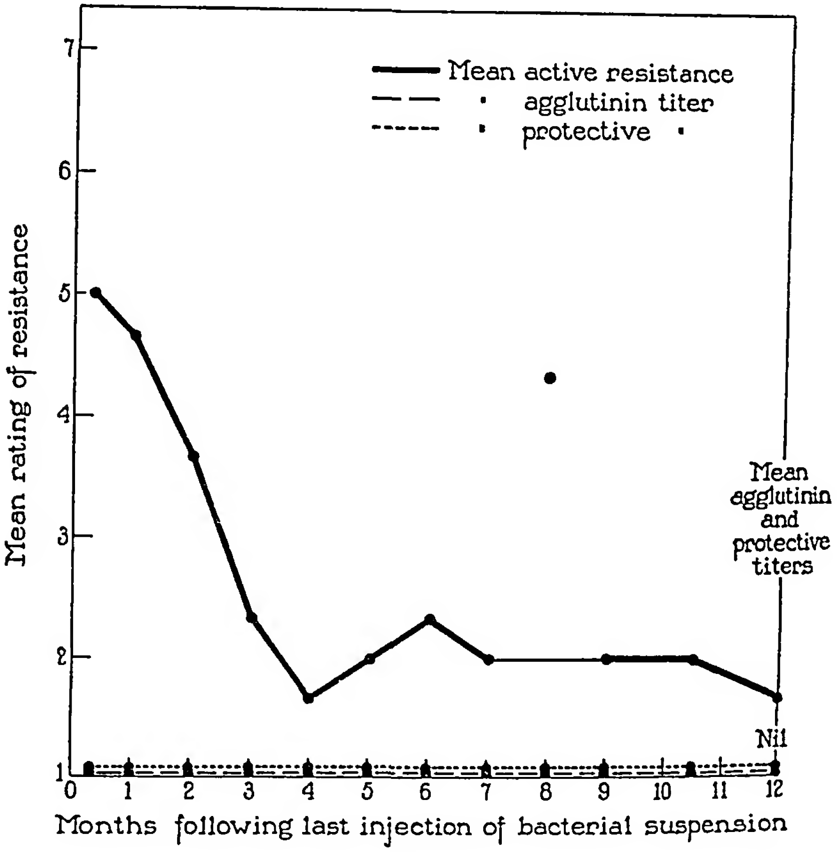
Degree and Duration of Active Resistance to Type II Pneumococcus Infection in Rabbits Following Intravenous Injections of Heat-Killed Suspensions of Pneumococci (Types I, II, and III)

Test interval	Rabbit No.	Serum antibodies		Test infection (Type II Pneumococcus)				
		Agglutinin titer	Protective titer	Febrile reaction		Extent of lesion	End-result	Grade of resistance
				Maximum	Duration			
days			*cc.*	*°F.*				
10	1	—	0.01	103.5	—	Widespread	S	6
	2	—	0.001	103.3	—	“	S	6
mos.								
1	1	—	0.000,001	104.3	2 days	“	S	5
	2	—	0.000,001	103.6		“	S	6
	3	—	0.000,001	103.7		“	S	6
2	1	—	0.000,001	105.2	2 days	“	S	5
	2	1–10	0.01	103.2	—	“	S	6
	3	—	0.01	104.0	1 day	“	S	5
3	1	—	0.001	104.2	1 day	“	S	5
	2	—	0.01	104.9	1 day	“	S	5
	3	—	0.001	105.5	1 day	“	S	4
4	1	—	0.000,1	103.5	—	“	S	6
	2	—	0.001	105.3	1 day	“	S	5
	3	—	0.001	104.8	1 day	“	S	5
5	1	—	0.001	103.8	—	“	S	6
	2	—	0.000,01	104.2	1 day	“	S	5
	3	—	0.001	104.0	—	“	S	5
6	1	—	0.01	102.7	—	Localized	S	7
	2	—	0.001	104.1	1 day	Widespread	S	5
	3	—	0.01	103.2	—	“	S	6
7	1	—	0.000,001	106.0	1 day	“	S	4
	2	—	0.01	104.8	1 day	“	S	5
	3	—	0.000,001	103.3	—	“	S	6
8	1	—	0.000,01	104.1	1 day	“	S	5
	2	—	0.000,1	104.3	1 day	“	S	5
	3	—	0.000,01	105.2	3 days	“	S	5
9	1	—	0.01	103.9	1 day	“	S	6
	2	—	0.001	104.2	1 day	“	S	5
	3	—	0.001	106.5	Until death	“	D 6	2
10½	1	—	0.000,001	104.1	1 day	“	S	5
	2	—	0.000,1	108.0	Until death	“	D 4	1
12	1	—	0.000,001	106.3	2 days	“	S	4
	2	—	0.000,1	105.6	2 days	“	S	4
	3	—	0.000,1	106.1	Until death	“	D 3	1

S = survival.

D = death, at indicated number of days after infective inoculation.

apparent that the high degree of immunity observed just after the conclusion of the immunizing procedure disappeared very rapidly, giving way to a second or intermediate phase in which a moderate degree of resistance was still present and in which most of the animals survived. This in turn gave way to a phase of very low resistance in which fully half of the animals died following infection and in which the average rating of resistance corresponded to Grade 2. It is presumed that this phase would eventually reach a final stage in which all animals would be completely lacking in ability to combat infection.



TEXT-FIG. 2. Charts of the degree and duration of active resistance to Type II pneumococcus infection and of specific serum antibodies in rabbits following intravenous injections of heat-killed suspensions of pneumococci (Types I, II, and III). The points represent the means of the various determinations at each test period.

Although a comparison of the means of the various determinations is suggestive of certain possible correlations between active resistance and antibody titers, a consideration of the data on some individual animals leads to a very confusing picture. From a determination of the passively transferable immunity, it is difficult to predict how an individual animal will react to infective inoculation.

Induced Active Resistance Against Type II Pneumococcus Infection

The results of test infections with Type II pneumococci are summarized in Table II. The means of the various determinations are shown in Text-fig. 2.

Only one rabbit in the series showed agglutinins against the Type II Pneumococcus. The protective titers of the sera of the individual rabbits were so varied that averages are without significance. No trend is apparent.

The active resistance at the first test period was somewhat lower than that with Type I but remained relatively unchanged for 9 months. The average rating for this entire period was around Grade 5, and there was much less individual variation than in the Type I pneumococcus infections. After 9 months the mean resistance dropped sharply and it is presumed that the protective mechanism which had previously been effective in maintaining the resistance was no longer sufficient to prevent serious infection. A comparison of the data on the individual cases reveals little correlation between protective titer of the serum and the grade of active resistance to infection.

It is difficult to understand why these animals maintained such high degrees of active resistance against Type II pneumococcus infection for this relatively long period of time. The results are somewhat paradoxical in view of the commonly accepted fact that Type II Pneumococcus is less antigenic than Type I. On the other hand, the virulence of Type II Pneumococcus is somewhat lower than that of Type I. Certainly it would seem that Type II Pneumococcus is not as invasive as Type I in the face of similar protective mechanisms.

Induced Active Resistance against Type III Pneumococcus Infection

The data on the rabbits which were given Type III pneumococcus test infections are presented in Table III and shown graphically in Text-fig. 3.

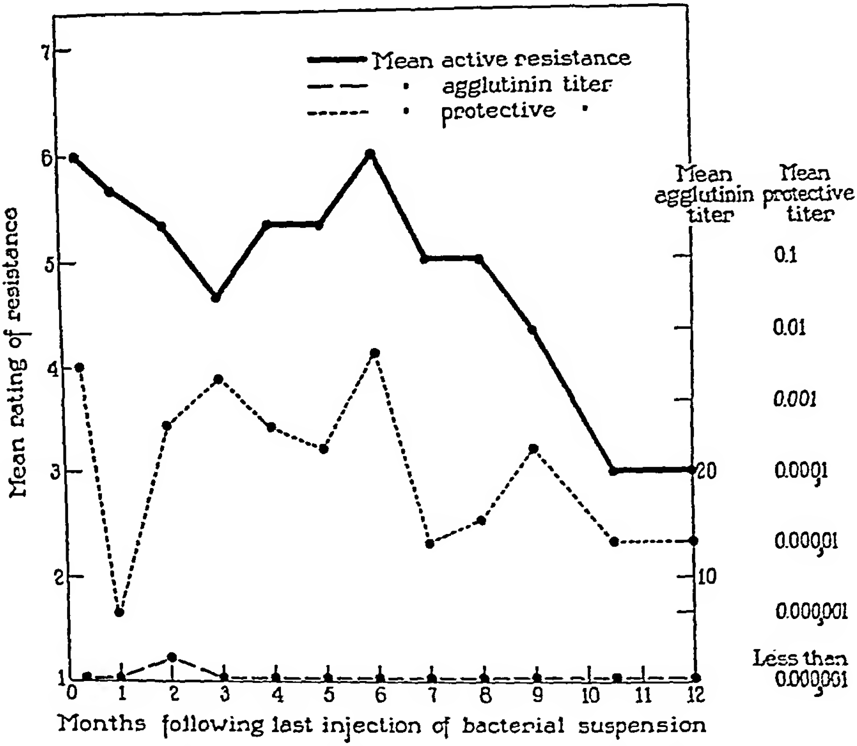
TABLE III

Degree and Duration of Active Resistance to Type III Pneumococcus Infection in Rabbits Following Intravenous Injections of Heat-Killed Suspensions of Pneumococci (Types I, II, and III)

Test interval	Rabbit No.	Serum antibodies		Test infection (Type III Pneumococcus)				
		Agglutinin titer	Protective titer	Febrile reaction		Extent of lesion	End-result	Grade of resistance
				Maximum	Duration			
days			*cc.*	*°F.*				
10	1	—	—	104.4	1 day	Widespread	S	5
	2	—	—	104.1	1 day	"	S	5
mos.								
1	1	—	—	105.8	1 day	"	S	4
	2	—	—	105.3	1 day	"	S	5
	3	—	—	104.3	1 day	"	S	5
2	1	—	—	105.5	3 days	"	S	4
	2	—	—	109.7	4 days	"	S	5
	3	—	—	107.3	Until death	"	D 5	2
3	1	—	—	106.0	" "	"	D 5	2
	2	—	—	106.9	13 days	"	S	3
	3	—	—	107.5	Until death	"	D 7	3
4	1	—	—	104.3	" "	"	D 3	1
	2	—	—	106.7	16 days	"	S	3
	3	—	—	104.2	Until death	"	D 2	1
5	1	—	—	107.9	" "	"	D 9	2
	2	—	—	105.9	" "	"	D 5	2
	3	—	—	106.6	" "	"	D 14	2
6	1	—	—	106.6	" "	"	D 12	2
	2	—	—	107.1	" "	"	D 7	2
	3	—	—	106.4	10 days	"	S	3
7	1	—	—	106.3	Until death	"	D 6	2
	2	—	—	106.3	6 days	"	S	3
	3	—	—	106.5	Until death	"	D 4	1
8	1	—	—	104.6	3 days	"	S	5
	2	—	—	104.7	2 days	"	S	5
	3	—	—	105.6	5 days	"	S	3
9	1	—	—	106.3	Until death	"	D 8	2
	2	—	—	106.1	" "	"	D 4	1
	3	—	—	106.5	6 days	"	S	3
10½	1	—	—	106.0	Until death	"	D 8	2
	2	—	—	107.5	" "	"	D 5	2
12	1	—	—	106.7	" "	"	D 3	1
	2	—	—	105.5	5 days	"	S	3
	3	—	—	106.4	Until death	"	D 2	1

S = survival.
D = death, at indicated number of days after infective inoculation.

No agglutinins or protective antibodies against Type III Pneumococcus were detected in the serum of any of these animals. Considering the short course of immunization and the well known low antigenicity of this type of Pneumococcus these results are not surprising.



TEXT-FIG. 3. Charts of the degree and duration of active resistance to Type III pneumococcus infection and of specific serum antibodies in rabbits following intravenous injections of heat-killed suspensions of pneumococci (Types I, II, and III). The points represent the means of the various determinations at each test period.

A fair degree of active resistance against infection with this type of Pneumococcus was shown during the 1st month. This fell off rather sharply and remained thereafter at a low level except for one irregularity at the 8th month at which time for some unknown reason two of the animals showed considerable resistance. These individual factors can never be entirely eliminated. This irregular finding is indicated by a point on the chart but is not connected with the remainder of the curve since it is obviously at great variance with the other findings.

Anti-R Titer and Resistance to Infection

In about fifty of these animals determinations were made of the anti-R titer of the sera. No correlation was found between these titers and the grade of active resistance or with other determined factors.

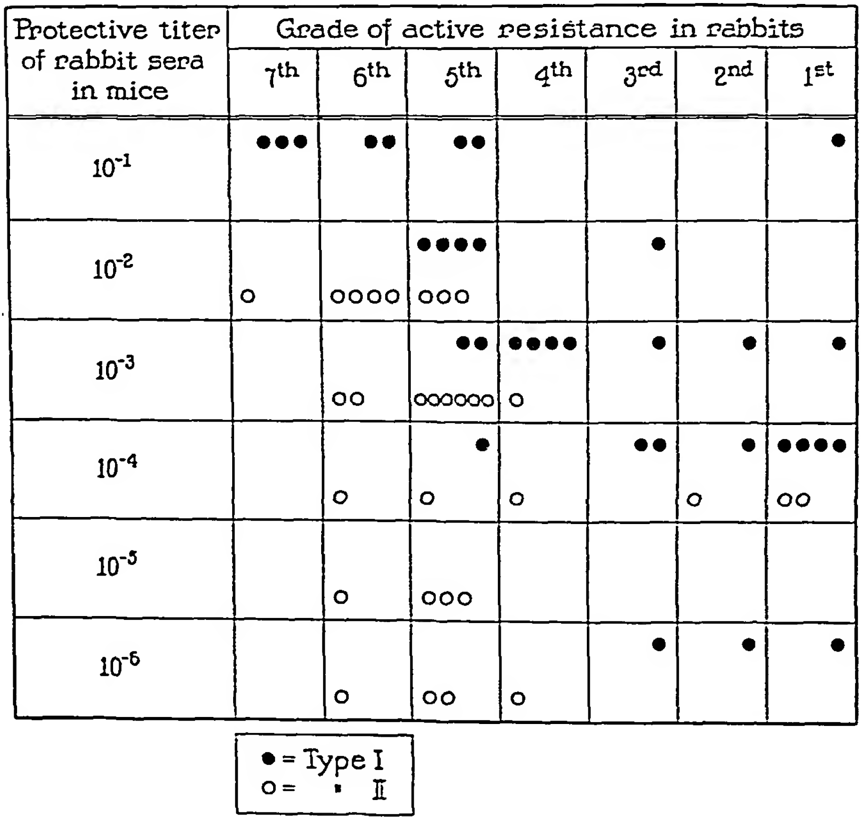
Correlation of Active Resistance and Antibody Titers

The lack of parallelism between the specific agglutinin titer and the mouse-protective titer in the same rabbit sera is not reckoned as a significant matter and may be largely accounted for by the fact that the mouse-protective test is much more sensitive than 'the agglutinin titration. It will be noted from the foregoing data that, in each instance in which agglutinins were demonstrated, the active resistance to infection was relatively high. The converse was not true.

Although there is some correlation between protective titer and the degrees of active resistance when the averages for any time period are considered, the suggestion of parallelism is less apparent when the data on the individual animals are studied. The results of the protection titrations in mice have been plotted against the grades of active resistance for each animal in the Type I and Type II series and are shown in Text-fig. 4. The results with the Type III infections have not been included since no protective antibodies were obtained with this immunization procedure.

It will be noted that on this chart the solid circles which represent the animals infected with Type I pneumococci occupy in general one section while the open circles which represent the animals infected with Type II pneumococci are oppositely placed. It would appear that the resistance to infection with one type of Pneumococcus is quite different from that exhibited against another type even though the sera of the rabbits may show equivalent potencies in the passive protection of mice. Thus, with high protective titers, animals show a wide range of degrees of resistance to Type I pneumococcus infection, whereas with widely varying protective titers, animals show relatively high resistance to Type II pneumococus infection. It is suggested that this may be accounted for in part by the differences in virulence between the two types, for it might be expected that the Type II Pneumococcus, possessing the lower virulence for normal rabbits, might also be less invasive in the face of similar protective mechanisms.

It may be pointed out that, in the individual instance, a high protective titer is not necessarily correlated with a high degree of active resistance, and *vice versa*. On the other hand, and especially with the Type I pneumococcus infections, there is a general correlation, since if one considers either the medians or the modes at the various levels of protective titers, it is apparent that the higher grades of active resistance may be roughly correlated with the higher levels of pro-



Protective titer of rabbit sera in mice	Grade of active resistance in rabbits						
	7th	6th	5th	4th	3rd	2nd	1st
10^{-1}	●●●	●●	●●				●
10^{-2}	○	○○○○	●●●● ○○○		●		
10^{-3}		○○	●● ○○○○○○	●●●● ○	●	●	●
10^{-4}		○	● ○	○	●●	● ○	●●●● ○○
10^{-5}		○	○○○				
10^{-6}		○	○○	○	●	●	●

● = Type I
○ = " II

TEXT-FIG. 4. The degrees of active resistance in relation to the specific protective titers in individual rabbits.

tective substances in the sera of the rabbits. The many exceptions to this parallelism tend to confuse the issue to such an extent that general conclusions cannot be drawn. It is possible that if all other factors entering into the phenomena of active resistance could be kept constant there would be an absolute parallelism between specific antibody titer and active resistance. Evidence is accumulating to the effect that there are many conditions which may serve to considerably modify active resistance due to specific immune factors, and that the manifestations of specific active resistance are largely conditioned by the adequacy of the non-specific factors and their capacity to function. The result of a test for active resistance is essentially the algebraic sum of all specific and constitutional factors. Another element which tends to confuse any theoretical parallelism is that the mouse-protective titration for passively transferable specific antibodies involves a second complex biological structure in which these same constitutional factors may be as varied as in the rabbit from which the serum was derived.

DISCUSSION

Since Type I, Type II, and Type III pneumococci differ in virulence for rabbits and in their antigenic capacity, it might be expected that certain differences might be encountered in the degree and duration of specific resistance brought about by immunization procedures. This has been shown to be the case. The height of the resistance immediately following immunization appears to be comparable to the relative antigenic capacity of the type of Pneumococcus. On the other hand, the duration of high resistance was short in the case of Types I and III but much longer in the case of Type II.

Although the animals were immunized with a suspension of pneumococci of three types, it is apparent that the results are characteristically different for each type used for infection.

These experiments emphasize the fact that survival and death serve as a poor index of the resistance against specific infection by the individual animal following immunization procedures, except in indicating a dividing line in the gradient of resistance.

At first examination the data presented might throw some weight against the prevalent conception of the rôle played by certain determinable antibodies in protection against infection. It must be borne in mind, however, that there are many limitations in determining outside of the animal body all of the factors which may take part in the increased resistance. There is at present no method of determining *in vitro* the sessile antibodies or other cellular factors which may play a significant rôle. An equally important fact is that the evaluation of circulating antibodies at the time of infective inoculation gives no index of the latent but conditioned potentiality of the animal body to produce quickly or to renew specific resistance factors after the stimulus of infection.

These facts may have some significance in the matter of active antipneumococcic immunization in man. It may be pointed out that the number of immunizing injections and the amount of bacterial material which was used correspond rather closely to the conditions employed in certain previous attempts at human immunization. While such an immunization procedure in rabbits does enhance greatly the possibility of the rabbit surviving any specific infection it does not prevent, after the 1st month, a considerable involvement which is generally accompanied by some form of systemic reaction. The increase of resistance induced in man by such a procedure might conceivably be greater since the natural resistance already present in man might be considerably greater than that in the rabbit.

SUMMARY

The degree and duration of the active resistance against pneumococcus infection have been determined in rabbits which received intravenous injections of a heat-killed suspension of pneumococci of the three principal types. The resistance induced by the immunization procedure varied in degree and in duration with each type of Pneumococcus.

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THE TITRATION OF YELLOW FEVER VIRUS IN STEGOMYIA MOSQUITOES*

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To workers engaged in the study of yellow fever it has always been a question of interest whether the virus multiplies in its insect host. Gay and Sellards (1) in 1927 plotted a conjectural curve showing an increase of virus in mosquitoes for 3 weeks after the ingestion of infectious blood; the initial increase was followed by a constant level. Recently, Davis (2), in discussing the early development of infectivity in stegomyia mosquitoes at temperatures of 30°C. and above, gave as a probable explanation the rapid multiplication of virus at those temperatures. The present series of titration experiments shows conclusively that in the highly efficient insect host, *Aëdes* (*Stegomyia*) *aegypti* (Linn.), the quantity of virus present never surpasses that originally ingested.

Methods

It was planned at first to titrate neurotropic yellow fever virus in mosquitoes which had fed on mice or on monkeys at a suitable interval after a massive inoculation with infected mouse brains. Various difficulties supervened. It was found that the neurotropic virus did not reach a high concentration in the blood stream of either species of animal. Feeding of mosquitoes on artificial mixtures of blood and infected mouse brains proved to be impracticable; never did enough insects feed to allow for an adequate series of titration exeriments. For detecting small quantities of virus in mosquito suspensions, the intracerebral route of injection into mice proved superior to the intraperitoneal route; however, irrespective of the route of injection, bacterial contaminants in the lower dilutions frequently

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* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation.

killed many animals. Filtration introduced an unknown, and probably variable, factor. In spite of the considerable expense involved, it was finally decided to work with viscerotropic yellow fever virus, Asibi strain, and to use *rhesus* monkeys as test animals.

Three series of titration experiments were performed. For the first series about 600 to 700 insects, and for each of the second and third series about 1,000 insects, never previously fed on blood, were allowed to engorge on an infected monkey. For convenience in handling they were distributed among four to six cages. At the beginning of each series all were approximately 2 weeks old. In each initial experiment, before the meal on infectious blood, 120 mosquitoes were weighed alive, 10 at a time, in celluloid catching tubes. In the first and third series honey and water remained in the cages until the hour of feeding; in the second series food and water had been removed about 16 hours beforehand. The infected (febrile) monkey was left in each cage for 1/2 hour. The amount of blood ingested was determined at the end of the feeding period from the increased weight of 100 mosquitoes intended for titration in the first experiment. For this, as well as for subsequent experiments, representatives were caught from all cages. Mosquitoes that had failed to take blood were destroyed. Except for such blood meals as will be mentioned in the protocols, the infected lots of mosquitoes received routinely only honey for food; wet cotton in each cage furnished water.

One hundred infected mosquitoes were titrated in nearly every experiment. For Experiment 5 of the first series only 90 mosquitoes were available; for Experiment 6 of the second series only 87 mosquitoes were still alive. The insects were anesthetized with chloroform at the time of catching; many of them were not actually killed until they were gound in the mortar. Except in the early experiments of the first series they were mashed into a paste with a few drops of undiluted normal monkey serum; to the paste was added 0.5 gm. of finely powdered pyrex glass (this material for grinding was chosen at the suggestion of Dr. J. H. Bauer). The glass had received a preliminary treatment with strong sulfuric acid, had been washed many times with distilled water, and had been dried in a porcelain dish over a flame. In small La Motte cups for testing hydrogen ion concentration, the addition of this powder to distilled water of pH 6.9 changed the reaction to pH 7.1. Grinding of the mosquitoes after the addition of powdered glass occupied about 3 minutes.

The lowest dilution in each case was 1:100 (except in the first series, where an error in calculation was made). Dilution was based on volume of blood ingested, determined from the weight, using the factor 1.06 as the approximate specific gravity of monkey blood. Included in the calculated total of dilution fluid were the few drops of whole serum already added before grinding; the rest of the fluid was 10 per cent normal monkey serum in physiological saline solution. Approximately one-half of this quantity was saved for rinsing the mortar and pestle. The total time required for grinding and diluting was 6 to 7 minutes. Dilutions in all experiments subsequent to the first one in each series were made as if the mosquitoes contained at the moment the original amount of blood ingested. That is, in a given series the amount of dilution fluid was the same in every experiment. The 1:100 suspension of ground mosquitoes was lightly centrifuged for 3 minutes. An attempt was made to have the rate of centrifugalization the same each time, but probably this uniformity was only approximate because of variations in strength of electric current. From the somewhat turbid supernatant fluid higher dilutions were made with 10 per cent normal monkey serum, using a clean sterile pipette for each successive dilution.

Monkeys, usually in duplicate, were inoculated subcutaneously with 1 cc. of each dilution. In no instance did an unnecessary delay occur; injections were begun within 1/2 hour of catching the mosquitoes. Temperatures of the animals were taken twice daily during the period of observation. At the end of about 3 weeks survivors were bled for a test of immunity, but were still kept under observation. Prolonged surveillance was necessary, because five monkeys died with typical yellow fever at 3 weeks, or more, after inoculation; the two latest deaths were at 29 and at 27 days, respectively. In all cases of death, confirmation of yellow fever was made by microscopical examination of liver sections. Sera from survivors were tested against neurotropic virus in mice. Animals whose sera gave protection, and those whose sera were inconclusive, were given test doses of potent viscerotropic virus as a further check on the presence of immunity. Those whose sera were definitely negative by the mouse test, and which survived an additional period of observation without fever, were considered suitable for use in later experiments.

Results of Mosquito Titrations in Monkeys

Series I (Begun February 15, 1932).—The average weight of 120 unfed mosquitoes chosen as a sample, 30 from each of four cages, was 2.805 mg. The average weight of blood ingested by 100 engorged mosquitoes used in the first experiment, was 1.25 mg.

Table I summarizes the results of titrations immediately following engorgement, and at intervals of 4, 14, and 28 days thereafter. The immediate end-point (first experiment) was at a dilution of 1:18,000-000. The end-point in the two succeeding experiments (at 4 and at 14 days) was at a dilution of 1:36,000. The end-point in the fourth experiment (at 28 days) was at a dilution of 1:1,800,000.

To account for the rise in titer from the third to the fourth experiment several factors had to be considered, which conceivably may have influenced results: (*a*) the passage of time (a fortnight between experiments); (*b*) a single meal on normal monkey blood 2 days before the fourth titration (3); (*c*) the addition in the fourth experiment of 3 drops

TABLE I

Mosquito Titrations, Series I

Dilution*	Experiment 1. Day of infective blood meal					Experiment 2. 4 days after infective blood meal					Experiment 3. 2 wks. after infective blood meal					Experiment 4. 4 wks. after infective blood meal†				
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity
1:90																a	+	+	8	
																b	+	+	6	
1:180	a	+	+	6		a	+	+	7		a	+	−	−	+	c	+	+	9	
											b	+	+	8		d	+	+	7	
1:1,800	b	+	+	5		b	+	+	8		c	+	+	7		e	+	+	7	
											d	+	+	8		f	+	+	8	
1:36,000	c	+	+	6		c	−	−	−	−	e	−	−	−	−	g	+	−	−	+
						d	+	+	7		f	+	−	−	+	h	+	−	−	+
1:180,000	d	+	+	7		e	−	−	−	−	g	−	−	−	−	i	+	−	−	+
	e	+	−	−	+	f	−	−	−	−	h	+	−	−	−					
1:450,000	f	+	+	7		g	−	−	−	−	i	+	−	−	−	j	+	+	9	
	g	+	+	5		h	−	−	−	−	j	−	−	−	−	k	+	+	9	
1:900,000	h	+	+	7		i	−	−	−	−	k	+	−	−	−	l	−	−	−	−
	i	+	+	7		j	−	−	−	−	l	−	−	−	−					
1:1,350,000	j	+	+	9		k	−	−	−	−	m	+	−	−	−	m	+	+	8	
	k	−	−	−	−	l	−	−	−	−	n	−	−	−	−					

1:1,800,000	l m	+ +	− +	− 6	−	m n	− −	− −	− −	− −	o p	+ −	− −	− −	− −	n	+	−	−	+‡
1:18,000,000	n o	− +	− +	− 14	−	o p	− −	− −	− −	− −	q	+	−	−	−	o	−	−	−	−
1:90,000,000	p	+	−	−	−	q	−	−	−	−	r	+	−	−	−					
1:180,000,000	q r	− −	− −	− −	− −	r s	− −	− −	− −	− −	s	−	−	−	−					
1:900,000,000	s t	+ +	− −	− −	− −	t u	− −	− −	− −	− −	t	−	−	−	−					
1:1,800,000,000	u v	− −	− −	− §	−	v	−	−	−	−	u	+	−	−	−					
	w	+	−	−	−															

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* An error of calculation in the first experiment brought dilutions to odd fractions. To make results comparable, the same process of dilution was used in all experiments. The two lowest dilutions are given to the nearest 10, and higher dilutions correspondingly in round numbers.

† In this Experiment 3 drops of undiluted normal monkey serum was added to the mosquitoes before grinding with powdered glass.

‡ Monkey died 6 days after the last fever without definite lesions of yellow fever. Serum at that time gave positive protection test in mice.

§ Died of dysentery.

of undiluted normal monkey serum to the mosquitoes before grinding with powdered glass.

A sufficient number of mosquitoes remained alive to test the protective value of whole serum. Therefore, a fifth experiment (not included in Table I) was performed. Ninety surviving mosquitoes were killed and distributed equally between two mortars. To one-half, whole serum was added before grinding; to the other half none was added. Both lots were diluted to 1:180,000 with 10 per cent normal monkey serum; and six monkeys were inoculated, three from each lot. Of the monkeys inoculated from the mosquito lot which had received an addition of whole serum before being ground, all three died with yellow fever. Of those inoculated from the duplicate lot of mosquitoes which had not been ground with serum, only one died, and the other two were not immunized. The result indicated that the addition of whole serum before grinding with powdered glass undoubtedly helped to preserve the virus.

Series II (Begun March 28, 1932).—The average weight of 120 unfed mosquitoes chosen as a sample, 20 from each of six lots, was 2.783 mg. The average weight of blood ingested by 100 engorged mosquitoes used in the first experiment was 1.534 mg. In each experiment 0.3 cc. of undiluted normal monkey serum was added to the mosquitoes before grinding.

Table II summarizes the results of titrations immediately following engorgement, and at intervals of 1, 3, 5, and 11 weeks thereafter. The immediate end-point (first experiment) was at a dilution of 1:1,000-000,000. 1 week later the highest dilution causing fatalities was 1:1,000,000. At 3 weeks the dilution 1:50,000,000 killed a monkey. At 5 weeks the highest infective dilution was also 1:50,000,000 for the mosquitoes which had been offered a blood meal four times in the preceding fortnight; for those which had ingested no blood, it was 1:1,000,000. Before feeding any lots between the experiments at 3 weeks and at 5 weeks, a thorough intermixture of mosquitoes was effected among Batches 1 and 4, 2 and 5, 3 and 6; this should have equalized the lots offered blood (Nos. 1 to 3) and those not offered blood (Nos. 4 to 6). During the succeeding 6 weeks no mosquitoes took blood. The combined survivors of all lots (87 mosquitoes) at 11 weeks after infection gave an end-point at a dilution of 1:1,000,000.

Series III (Begun May 30, 1932).—The average weight of 120 unfed mosquitoes chosen as a sample, 20 from each of six lots, was 2.47 mg. The average weight of blood ingested by 100 engorged mosquitoes used in the first experiment was 1.13 mg. In each experiment 0.35 cc. of undiluted normal monkey serum was added to the mosquitoes before grinding.

Table III summarizes the results of titrations immediately following engorgement, and at intervals of 4 days, and of 6 weeks thereafter. The immediate end-point (first experiment) was at a dilution of 1:1-000,000,000. 4 days later the end-point was at 1:10,000,000. After 6 weeks it had risen to 1:100,000,000, both for the mosquitoes which had been offered six blood meals in the interval between experiments, and for those which had received only honey and water in the meanwhile.

Mosquito Titrations in Mice

Athough, as previously stated, none of the titrations of neurotropic virus was satisfactory, the least unsatisfactory experiment may be cited as confirmatory evidence of a reduction of titratable virus in infected mosquitoes.

On Dec. 3, 1931, there was prepared a 33 1/3 per cent suspension of infected mouse brains in diluting fluid (10 per cent normal monkey serum). 1 cc. of the mixture was injected intraperitoneally into each of twelve mice (Groups R547 and R548). Between 2 and 3 hours later Mosquito Lot 675, which had been without food or water for 24 hours, was allowed to feed. From the weights of 100 engorged mosquitoes the average blood meal was calculated at 2.3 mg. With these insects six dilutions were made, the lowest 1:25, the highest, 1:100,000; and each dilution was injected intracerebrally into a group of six mice. Deaths occurred in every mouse group except the last. From the group inoculated with dilution 1:10,000, a positive subinoculation was obtained. The infectivity of the mice upon which mosquitoes fed was tested immediately after the blood meal by the injection of pooled serum from three of these into twelve other mice, all of which died in a typical manner on the 4th and 5th days thereafter.

On Dec. 17, a fortnight after the infective blood meal, 106 of the mosquito lot were killed for the second experiment. Nine dilutions were made, the lowest 1:25, the highest 1:1,000,000, and each dilution was injected intracerebrally into a group of six mice. Deaths occurred among the mice inoculated with dilutions 1:25, 1:100, and 1:500. Subinoculated mice all died early, indicating bacterial contamination. However, even if virus were present up to dilution 1:500, the titer had dropped to 5 per cent of that in the first experiment.

TABLE II

*Mosquito Titrations, Series II**

Dilution	Experiment 1. Day of infective blood meal				
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity
1:100	a	+	+	5	
1:1,000					
1:20,000					
1:100,000	b	+	+	8	
1:250,000	c	+	+	5	
1:500,000	d	+	+	5	
	e	+	+	5	
1:750,000	f	−	+	5	
	g	+	+	8	
1:1,000,000	h	−	+	5	
	i	+	+	6	
1:10,000,000	j	+	+	6	
	k	+	−	−	+

Dilution	Experiment 2. 1 wk. after infective blood meal				
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity
1:100	a	+	−	−	+
1:1,000	b	+	+	7	
1:20,000	c	+	+	7	
1:100,000	d	+	+	11	
	e	+	+	6	
1:250,000	f	+	+	6	
	g	−	+	8	
1:500,000	h	+	+	8	
	i	+	−	−	+
1:750,000	j	+	+	10	
	k	+	+	9	
1:1,000,000	l	+	−	−	−
	m	+	+	7	
1:10,000,000	n	+	−	−	−
	o	+	−	−	−

Dilution	Experiment 3. 3 wks. after infective blood meal				
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity
1:100	a	+	+	7	
1:1,000	b	−	−	‡	
1:20,000	c	−	+	6	
1:100,000	d	+	+	7	
1:250,000	e	+	+	9	
	f	+	+	6	
1:500,000	g	+	+	8	
	h	+	+	6	
1:750,000	i	−	+	6	
	j	+	+	7	
1:1,000,000	k	+	+	6	
	l	+	+	7	
1:10,000,000	m	−	+	7	
	n	+	−	−	−

Dilution	Experiment 4. 5 wks. after infective blood meal†				
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity
1:100					
1:1,000					
1:20,000	a	+	+	5	
1:100,000	b	+	−	−	+
1:250,000	c	+	+	5	
1:500,000	d	−	−	−	−
	e	+	+	7	
1:750,000	f	+	+	8	
	g	+	+	8	
1:1,000,000	h	+	+	8	
	i	+	+	8	
1:10,000,000	j	+	+	8	
	k	+	+	8	

Dilution	Experiment 5. 5 wks. after infective blood meal†				
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity
1:100					
1:1,000					
1:20,000					
1:100,000	a	+	+	8	
1:250,000	b	+	−	−	−
1:500,000	c	+	+	11	
	d	+	+	9	
1:750,000	e	−	+	9	
	f	+	+	17	
1:1,000,000	g	+	+	9	
	h	+	+	7	
1:10,000,000	i	−	−	−	−
	j	−	−	−	−

Dilution	Experiment 6. 11 wks. after infective blood meal				
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity
1:100					
1:1,000					
1:20,000	a	+	+	8	
1:100,000	b	+	+	8	
	c	−	+	8	
1:250,000	d	−	+	8	
	e	−	−	‡	
1:500,000	f	−	+	6	
	g	−	+	8	
1:750,000	h	+	+	17	
	i	+	+	16	
1:1,000,000	j	−	+	12	
	k	+	+	14	
1:10,000,000	l	−	−	−	−
	m	−	−	−	−

1:50,000,000	l	+	+	14		p	+	−	−	−	o	+	−	−	−	l	+	+	12		k	−	−	−	−	n	−	−	−	−
	m	+	+	12		q	+	−	−	−	p	+	+	27		m	+	+	6		l	−	−	−	−	o	−	−	−	−
1:100,000,000	n	−	+	5		r	+	−	−	−	q	−	−	−	−	n	−	−	−	−	m	+	−	−	−	p	−	−	−	−
	o	+	+	6		s	−	−	−	−	r	−	−	−	−	o	−	−	−	−	n	−	−	−	−	q	−	−	−	−
1:250,000,000																p	−	−	−	−										
1:500,000,000	p	−	−	−	−	t	+	−	−	−	s	+	−	−	−	q	−	−	−	−	o	−	−	−	−					
	q	−	−	−	+	u	+	−	−	−						r	−	−	−	−	p	−	−	−	−					
1:1,000,000,000	r	+	+	8		v	+	−	−	−	t	−	−	−	−	s	−	−	−	−	q	−	−	−	−					
	s	−	−	−	−	w	−	−	−	−																				
1:10,000,000,000	t	−	−	−	−	x	−	−	−	−	u	+	−	‡							r	−	−	−	−					
	u	−	−	−	−																									
1:100,000,000,000	v	−	−	−	−																									

* In each experiment 0.3 cc. of undiluted normal monkey serum was added to the mosquitoes before grinding.

† Mosquitoes used in Experiment 4 of this series had been offered a blood meal upon four occasions in the fortnight following Experiment 3. Mosquitoes in Experiment 5 were control mosquitoes which had not taken blood since the original infective feeding. Many non-immunizing fevers, particularly in Experiments 2 and 3, were caused by tuberculosis.

‡ Died of intercurrent disease.

TABLE III

*Mosquito Titrations, Series III**

Dilution	Experiment 1. Day of infective blood meal					Experiment 2. 4 days after infective blood meal					Experiment 3. 6 wks. after infective blood meal†					Experiment 4. 6 wks. after infective blood meal†				
	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity	Monkey	Fever	Death from yellow fever	Day of death following inoculation	Subsequent immunity
1:100,000						a	+	+	7		a	+	+	7		a	−	+	7	
1:250,000						b	+	+	9		b	−	+	9		b	−	+	7	
						c	+	+	8											
1:500,000	a	+	+	8		d	+	+	14		c	−	+	7		c	+	+	14‡	
						e	+	+	7		d	−	+	7		d	+	+	5	
1:750,000	b	+	+	6		f	+	+	13		e	−	+	8		e	−	+	8	
	c	+	+	6		g	+	+	5		f	−	+	9		f	+	+	14	
1:1,000,000	d	+	+	9		h	+	+	5		g	−	+	6		g	−	+	11	
	e	+	+	11		i	+	+	5		h	+	+	17		h	−	+	7	
1:10,000,000	f	+	+	9		j	+	+	10		i	+	+	18		i	+	+	9	
	g	+	+	11		k	−	−	−	−	j	−	+	22		j	−	+	−	+
1:50,000,000	h	+	+	5		l	−	−	−	−	k	−	−	−	−	k	+	+	18	
	i	+	+	7		m	−	−	−	−	l	−	−	−	−	l	−	−	−	−

1:100,000,000	j	+	+	7		n	−	−	−	−	m	−	−	−	−	m	+	+	21	
	k	+	−	−	+	o	−	−	−	−	n	+	+	29		n	−	−	−	−
1:500,000,000	l	+	+	15		p	−	−	−	−	o	−	−	−	−	o	−	−	−	−
	m	+	+	24		q	−	−	−	−										
1:1,000,000,000	n	+	+	6		r	+	−	−	−	p	−	−	−	−	p	−	−	−	−
	o	−	−	−	−															
1:10,000,000,000	p	−	−	−	−	s	−	−	−	−										
	q	−	−	−	−															
1:100,000,000,000	r	−	−	−	−															

* In each experiment 0.35 cc. of undiluted normal monkey serum was added to the mosquitoes before grinding.

† Mosquitoes used in Experiment 3 had been allowed to feed on monkey blood six times in the interval following Experiment 2. Mosquitoes used in Experiment 4 had taken no blood since their original infective feeding.

‡ Death complicated by peritonitis. Midzonal necrosis and intranuclear inclusions present in liver.

DISCUSSION

In 1930 Bauer and Mahaffy (4) and Aragão and da Costa Lima (5) reported titrations of yellow fever virus in infected mosquitoes. One of the experiments of the latter authors may be cited. Three stegomyia mosquitoes which had taken an infective blood meal 1 month previously were ground thoroughly in a mortar and were suspended in 10 cc. of distilled water. From this first dilution 1 cc. was added to 9 cc. of distilled water; succeeding dilutions were similarly made to a total of six. A monkey became infected following the injection of 1 cc. from the sixth dilution. As the authors remark, if the three mosquitoes had occupied a volume of 1 cc., the final dilution would have been in the neighborhood of 1:1,000,000. However, the mosquitoes probably weighed about 8 mg.; hence the final dilution was approximately

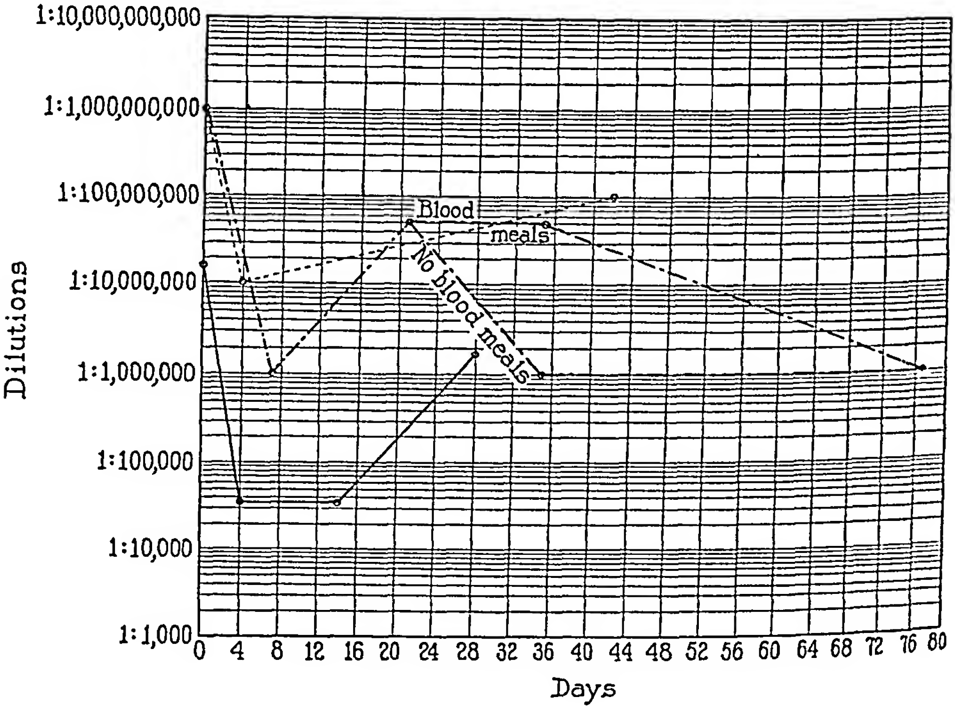

Chart 1. End-points in mosquito titrations.

———— Titrations first series.
—·—·— Titrations second series.
-------- Titrations third series.

1:125,000,000. Based on the amount of infectious blood originally ingested, the final dilution was probably between 1:150,000,000 and 1:300,000,000. This is a remarkable titer, considering that distilled water was used as the diluting fluid. Nothing is said in the protocol about filtration or centrifugalization to eliminate gross particles from the first dilution (original suspension).

The experiments reported in the present paper probably leave some room for criticism. However, they were as nearly comparable one with another as circumstances permitted. Perhaps the greatest source of error was in centrifugalization of the 1 per cent suspension. In the first experiment of each series all of the virus was contained in freshly ingested blood and was affected scarcely at all by centrifuging. In succeeding experiments it is very probable that some intracellular virus was not released by grinding but was carried down in the debris removed by centrifugalization. This would account in part for the drop in titer during the first week or two after an infective blood meal. A good part of the drop in titer after the first titration is doubtless due also to loss of virus by defecation.

If the preceding explanation of an increase in titer be not acceptable, it is necessary to postulate (*a*) gross differences in technique between experiments (perhaps errors in dilution), or (*b*) differences in susceptibility of test animals, or (*c*) wide variation in the amount of virus originally ingested by the mosquitoes, or (*d*) actual multiplication of virus, or (*e*) a biological change in the virus, rendering it more virulent. Injection of mosquitoes at any interval after a meal on infectious blood produces yellow fever. This fact has been amply verified by many workers and has been interpreted as ruling out a life cycle of the virus in mosquitoes. The possibility remains that part of the virus undergoes a cyclical change, or that all undergoes such a change, but at different rates or at different periods, so that at any given time there is some infective virus present. These are theoretical considerations lacking experimental evidence.

In spite of the shortcomings in technique, the experiments indicate that probably some actual loss of virus occurred soon after the infective blood meal. It seems certain that at no time was there present in the mosquitoes a greater amount of virus than the quantity originally ingested.

Spencer and Parker (6) showed that fresh blood activated, or increased the virulence of, Rocky Mountain spotted fever virus in fasting ticks; it is possible that in their experiments the virus (*Rickettsia*) multiplied also.

Dyer, Workman, Ceder, Badger, and Rumreich (7) proved to their satisfaction that the virus of endemic typhus undergoes an enormous multiplication in infected fleas (*Xenopsylla cheopis*). It is probable that all *Rickettsiae* multiply in the arthropod hosts to which they are adapted. Microscopical studies support this view. The behavior of yellow fever virus in the stegomyia mosquito illustrates once more the marked differences between a filterable virus *sensu restricto* and *Rickettsiae*.

Huff (8) has amplified the old classification of insect transmission of disease ("biological" and "mechanical") into four categories: cyclo-propagative, cyclo-developmental, propagative, and mechanical. The behavior of yellow fever virus necessitates still another group, because apparently neither propagation nor cyclical change takes place in the mosquito host; however, an incubation period is obligatory. Evidently migration to the salivary glands occurs during this period. Perhaps this type of transmission might be called "delayed mechanical."

The titer of 1:1,000,000,000 obtained in the first experiment both of Series II and of Series III, when mosquitoes containing freshly ingested blood-virus were used, confirms the work of Bauer (9). The latter produced yellow fever in *rhesus* monkeys with comparable dilutions of infectious blood.

These experiments prove that a stegomyia mosquito may ingest from 1 to 2 million lethal doses of yellow fever virus. An insect which has reached the stage of infectivity may still contain in its body 20,000, or more, lethal doses of virus.

Only two monkeys among survivors of all experiments became immune without showing a fever at some time during the period of observation. Monkey II 1 q had received an injection of mosquito dilution 1:500,000,000; Monkey III 4 j had received a dilution of 1:10,000,000. On the other hand, many animals died of typical yellow fever without having had a demonstrable febrile reaction (104°F., or higher); it is quite possible that transient fevers occurred at night in some of these monkeys.

SUMMARY

Titrations were made of yellow fever virus in stegomyia mosquitoes, using *rhesus* monkeys as test animals. It was found that:

(*a*) The average mosquito immediately after engorging on highly infectious blood contained between 1 and 2 million lethal doses of virus. The titer of freshly ingested blood was as high as 1 billion lethal doses of virus per cubic centimeter.

(*b*) During the fortnight succeeding a meal on infectious blood there occurred a reduction of titratable virus to not more than 1 per cent of that present in the freshly fed insects.

(*c*) The titer was somewhat higher at later periods. This rise in titer signified possibly not a multiplication, but merely an increase of extracellular virus and of that easily freed by grinding to a titratable form.

(*d*) At no later stage did the quantity of titratable virus equal that demonstrable in freshly fed insects.

Comment by Hugo Muench

In experiments such as the foregoing it is probably best to use as end-point the dilution at which one-half the animals die or are affected. In this way the entire group of animals used at different dilutions may in a sense be regarded as one sample, on the assumption that those living at a given dilution would have lived at a lower one, and *vice versa*. This method is a recognized statistical procedure and has the advantage of making the end-point a great deal less variable. The results of using the 50 per cent end-point are given below.

50 Per Cent Mortality

Series I	
Experiment 1	1:1,800,000
Experiment 2	1: 9,000
Experiment 3	1: 1,800
Experiment 4	1: 63,000
Series II	
Experiment 1	1:170,000,000
Experiment 2	1: 500,000
Experiment 3	1: 9,500,000
Experiment 4	1: 10,000,000
Experiment 5	1: 2,500,000
Experiment 6	1: 2,500,000

Series III	
Experiment 1	1:700,000,000
Experiment 2	1: 7,000,000
Experiment 3	1: 28,000,000
Experiment 4	1: 28,000,000

On comparing the figures in this tabulation with those of the text, the following main differences will be seen: (1) when the 50 per cent mortality point is used, the secondary rise is not nearly so pronounced, although it is uniformly present and is probably real. (2) The difference between the two sets of mosquitoes in Series II (those receiving and those not receiving additional blood meals) almost disappears. It is doubtful whether there is any such real difference. This would agree with the findings of the authors in Series III.

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VACCINATION WITH HEAT-KILLED AND FORMALINIZED TUBERCLE BACILLI IN EXPERIMENTAL TUBERCULOSIS

By ROBERT M. THOMAS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, May 27, 1933)

In recent years a number of antigens and vaccines have been prepared with the use of formaldehyde. Following the work of Ramon (1, 2), who demonstrated that formaldehyde reduces the toxicity of certain animal and vegetable toxins while maintaining their antigenic properties, Wherry and Bowen (3) and subsequently Wherry *et al.* (4) showed that certain bacterial vaccines could be "detoxified" by this method. Petragnani (5) prepared a vaccine from tubercle bacilli by the use of formaldehyde, for which it was claimed that guinea pigs were made resistant to subsequent infection with tuberculosis and also that if the vaccine were allowed to settle into two portions—sediment and supernatant fluid—, the supernatant fluid could be used for diagnostic tests in a manner similar to tuberculin. While no substance resembling a true toxin has been isolated from the tubercle bacillus or from the medium upon which it has been grown, it was thought that under the action of formaldehyde certain of the undesirable reactions to tubercle bacillus vaccines might be eliminated. The following experiments were undertaken to compare the properties of a vaccine prepared by the use of formaldehyde with the properties of a heat-killed vaccine. Comparisons were made of the immunizing power, of the skin-sensitizing power, and of the rapidity of absorption of the two vaccines. A study of the lesions produced by the intravenous injection of heat-killed bacilli was made as a result of some observations on rabbits vaccinated in this manner.

Material and Methods

Rabbits of about 2 kilos in weight were selected for use in the longevity experiment. They were kept under observation for a short period of time before any

Series III	
Experiment 1	1:700,000,000
Experiment 2	1: 7,000,000
Experiment 3	1: 28,000,000
Experiment 4	1: 28,000,000

On comparing the figures in this tabulation with those of the text, the following main differences will be seen: (1) when the 50 per cent mortality point is used, the secondary rise is not nearly so pronounced, although it is uniformly present and is probably real. (2) The difference between the two sets of mosquitoes in Series II (those receiving and those not receiving additional blood meals) almost disappears. It is doubtful whether there is any such real difference. This would agree with the findings of the authors in Series III.

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Material and Methods

Rabbits of about 2 kilos in weight were selected for use in the longevity experiment. They were kept under observation for a short period of time before any experimental procedures were started, while records of their blood cells and their weights were made. The vaccines were all made from a strain of bovine tubercle bacilli known as B-1. The formalin-killed vaccine was prepared by suspending the organisms in an aqueous solution of 0.4 per cent formaldehyde and 0.9 per cent sodium chloride, and incubating at 37°C. for 10 days. After incubation the suspensions were centrifuged and the organisms washed in distilled water three times and resuspended in 0.9 per cent sodium chloride solution. The heat-killed vaccine was prepared by suspending the organisms in 0.9 per cent sodium chloride solution and heating to 70° for 1 hour. Samples of both vaccines were planted on Petroff's egg media and also injected intravenously into normal rabbits as a control of the sterility of the preparations. The results in each case were negative for living tubercle bacilli.

The first experiment was designed to compare the longevity of tuberculous rabbits which had been vaccinated with heat-killed vaccine with that of tuberculous rabbits vaccinated with formalin-killed vaccine. Rabbits which had received no vaccine were inoculated with the same dose of tubercle bacilli at the same time. The vaccine was given intravenously in two series of injections. The first series, 4 months before inoculation, consisted of seven daily injections of 0.3 mg. each. The second series of injections, 1 month before inoculation, consisted of ten doses of 1.3 mg. each, given at 2 day intervals. Ten rabbits received the formalin-killed vaccine and five rabbits received the heat-killed vaccine, while five animals were kept as controls. Of the ten animals vaccinated with formalin-killed bacilli, two died in the 2 month interval between the two series of injections and at autopsy showed severe snuffles and a non-tuberculous pneumonia. A third animal died after the completion of the two series of injections and before inoculation, and at autopsy showed snuffles and a non-tuberculous pneumonia. Two rabbits were added before the second series of injections to replace the two that had died, so that nine rabbits altogether received intravenous formalin vaccine and were inoculated. In the case of the five rabbits which were given heat-killed bacilli, one died with severe snuffles and a non-tuberculous pneumonia, while three others died during the course of the second series of injections. These at autopsy showed a massive consolidation of both lungs with a tubercular pneumonia, without any evidence of secondary infection, and in the livers and spleens a few epithelioid cells were found on microscopic examination. As a result of the loss of seven rabbits out of the fifteen vaccinated, the intravenous route of injection was discarded in favor of the subcutaneous route, and five normal rabbits were inoculated along with the nine animals which had received formalin-killed vaccine intravenously. These were all given a series of subcutaneous injections of formalin vaccine during the first 4 months of the disease, starting with the day of inoculation and repeated at monthly intervals up to the 4th month when injections were given at weekly intervals. Eight doses of 0.1 mg. were given in all.

A second experiment was started later with two groups of ten rabbits, one vaccinated with heat-killed bacilli and the other with formalin-killed bacilli subcutaneously at 10 day intervals before inoculation. Five doses of 0.1 mg. were given in total. One of the rabbits injected with formalin-killed vaccine died just before inoculation.

RESULTS

The mean longevity of each of the four groups of vaccinated rabbits is shown in Chart 1. The longevity of the unvaccinated, tuberculous controls is expressed as 100 per cent and the longevity of the vaccinated animals compared with this figure. It will be seen that the rabbits which were vaccinated subcutaneously with heat-killed bacilli survived much longer than their controls, while in none of the other groups was the survival as long as that of their controls.

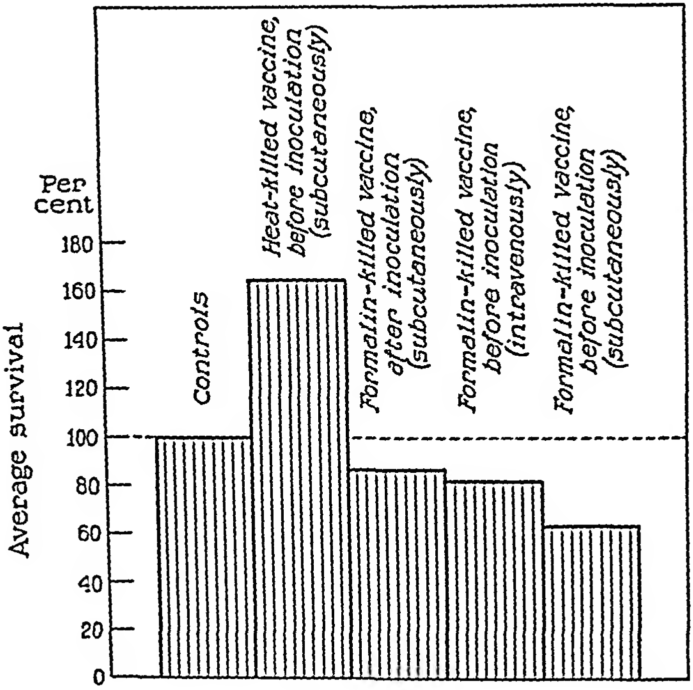

CHART 1

Chart 2 shows the distribution of deaths by months after infection in the two groups which received vaccine subcutaneously and in the group of control rabbits which were inoculated at the same time. It will be seen that while the deaths in the group vaccinated with formalin-killed organisms were distributed in a manner similar to that of the controls, the group which was vaccinated with the heat-killed organisms showed a much greater survival. It is noteworthy that in each case some rabbits died in the first phase of the disease, that is, during the first 3 months: in the case of the controls, 25 per cent; in the case of the formalin-killed vaccine group, 44 per cent; and in the case of the heat-killed group, 20 per cent. The effect of the vaccine was thus more apparent in slowing down the chronic phase of the disease than it was in preventing deaths from occurring in the first acute phase of the disease.

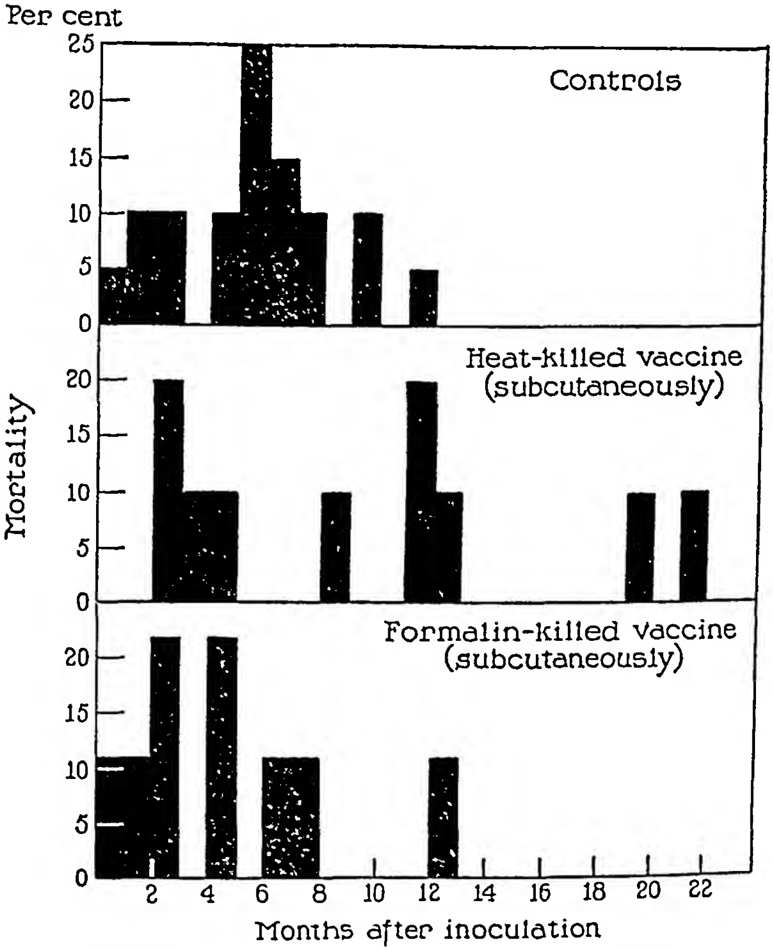

CHART 2

Differences in the course of the disease between the treated and control groups were sought, but in each case the two groups were similar, in so far as direct comparison was possible. In comparing the lesions of one group of rabbits with those of another group, control animals of equal or nearly equal survival must be chosen for comparison, as the extent and the nature of the lesions found in untreated tuberculous animals during successive months after infection vary greatly. Thus, despite the increased longevity of the first group, vaccinated subcutaneously with heat-killed bacilli, the lesions found at autopsy were similar both in extent and type to those found in control animals of the same survival. The changes which occurred in the blood cells, the variations in weight, and the autopsy findings were all typical of the disease as it is seen in untreated control rabbits. Hence it would appear that acquired resistance to tuberculosis in rabbits manifests itself in the ability of the treated animals to retard the rate of progress of the disease, rather than in their ability to modify or heal the lesions.

The rate of absorption of the two vaccines when injected under the skin appeared to be similar. Both produced a soft, fluctuant mass when introduced in this manner, which required 1 or 2 weeks for absorption. Sections of these masses showed them to be typical of tubercular tissue undergoing caseation.

The skin-sensitizing ability of the two vaccines appeared to be equal. Two groups of guinea pigs were injected intraperitoneally with three doses of 2.5 mg. of heat-killed and formalin-killed vaccine respectively. 36 days later, all of the guinea pigs were found to be markedly sensitive to tuberculoprotein (MA-100), and no significant difference between the two groups could be detected.

The failure of the formalin-killed vaccine to induce any appreciable resistance to tuberculous infection in rabbits is evident from the foregoing experiments. Whether the washing to which the organisms were subjected after incubation with the formalin removed any antigenic substance, or whether the formalin specifically destroyed the antigenic substance is problematical. Since the main water-soluble constituents of the tubercle bacillus, namely tuberculopolysaccharide and tuberculoproteins, are not known to possess any immunizing properties (aside from the skin-sensitizing ability of the tuberculoprotein), it was felt that washing would have no deleterious effect.

One is forced to return to the conclusion, reached before by many workers, that the antigenic properties of a tubercle bacillus vaccine are easily destroyed by chemical manipulation and are retained when the vaccine is prepared by gentle heating.

Pathology of Lesions Caused by Heat-Killed Vaccine

The close resemblance of the lesions found in the three rabbits which died during the course of intravenous injections of heat-killed vaccine, to those lesions found in rabbits inoculated intravenously with living bacilli suggested a further study of the effects of inoculation with dead organisms.

Ten rabbits were given two series of intravenous injections of heat-killed vaccine and used for this study. One animal was sacrificed just before the beginning of the second series of injections, and subsequent rabbits were sacrificed after the second, third, fifth, sixth, and seventh doses of the second series of injections. One rabbit was allowed to live for 2 months after the second series of injections and was then sacrificed. The blood cells of two of the rabbits were followed at frequent intervals during the second series of injections.

In the lung of the animal which was sacrificed after two doses of vaccine, the alveoli and smaller bronchioles showed a reaction which consisted of polymorphonuclear leucocytes, monocytes, and epithelioid cells, and some red cells and fibrin. In the lung of the rabbit sacrificed after the third dose, the reaction of polymorphonuclear leucocytes was less marked and there were more epithelioid cells; the lung at this time showed the beginning of a tubercular pneumonia; after the fifth, sixth, and seventh doses the lungs were massively consolidated with a tubercular pneumonia, the air sacs being filled with epithelioid cells and epithelioid giant cells; no polymorphonuclear reaction or fibrinous exudate was seen. There was no congestion of the capillaries of the lung. The lungs at autopsy were increased in consistency and on cut surface showed a thick creamy exudate, which when examined by the supravital method proved to be made up of epithelioid cells and monocytes. The rabbit which was sacrificed 2 months after the last injection showed in the lung no pneumonic lesions, but a number of isolated tubercles, scattered rather uniformly throughout the entire parenchyma of the lung. These tubercles were made up of epithelioid cells which were degenerating, showing vacuolization and degeneration of their cytoplasm, and in some cases nuclear degeneration. One large, confluent tubercle showed slight caseous changes in the center with a few polymorphonuclear leucocytes. Other tubercles were heavily infiltrated with small lymphocytes and plasma cells. The spleens of these animals were relatively free from involvement. In one or two cases epithelioid cells were seen in the spleen, in the supravital technique; in the liver, however, the presence of epithelioid giant cells in small numbers was the rule.

The blood counts of the rabbits were followed carefully throughout the second series of injections. During the 1st day there was a leucopenia, the total white count going as low as 1,150 cells. On the 2nd day there was a recovery, with a leucocytosis, the total white blood count going to 13,200 cells. During the first 2 days, however, the monocytes remained at very low levels, the leucocytosis of the 2nd day consisting of a polymorphonuclear increase; the 3rd day showed in all cases a rise in monocytes, which was accompanied by the appearance of stimulated monocytes and epithelioid cells. These remained high until the 5th and 6th days after which they receded to practically normal levels. The coincidence of the appearance of stimulated monocytes and epithelioid cells in the blood stream with the production of a massive tubercular pneumonia is very clearly shown to occur in the disease produced by intravenous inoculation of living organisms, the two phenomenona occurring at about the 30th day after infection (6).

From the foregoing observations it would appear that the sequence of pathologic changes which occur after the injection of living tubercle bacilli intravenously into rabbits is simulated very closely following the injection of dead organisms. The one characteristic feature of the lesions produced by the dead organisms is their striking uniformity throughout the entire lung. The epithelioid cells are usually in the same stage of development or degeneration, depending upon the time at which the lesions are examined. In the lungs of rabbits infected with living bacilli, tubercles which are practically adjacent to one another may show widely differing characters. One may be judged as relatively recent, while another may be obviously of long standing, possibly calcified. On the other hand, if the tissues of rabbits which have been inoculated intravenously with living tubercle bacilli 2 or 3 months previously are examined, lesions in the lungs and spleen will be found which are regressing. This is seen much more prominently in the spleen than in the lung. Within these lesions the presence of polymorphonuclear leucocytes is not the rule, as in caseation; there may be no accessory cells within the lesions, while the periphery of the area may show an infiltration of lymphocytes, in contrast to an area of caseation, where the center may be infiltrated with leucocytes and the periphery ill defined.

If, as it appears, this process of regression and ultimate absorption of tuberculous lesions occurs without the intervention of caseation, the parenchyma of the involved organ should eventually be found to have returned to the normal state. That this occurs in the spleens of tuberculous rabbits, there is little doubt. In the lungs, where the disease regularly progresses in rabbits, there is evidence that both processes, caseation and regression, occur simultaneously. Following intravenous injection of living organisms, a widespread tuberculous pneumonia develops, which reaches its height at the end of the 1st month, while if animals are sacrified 2 or 3 months after inoculation, the disease is confined to discrete tubercles scattered throughout the lung. Sections show the parenchyma of the lung away from these tubercles to be apparently normal, while the lesions themselves show the greatest diversity in type, consisting of tubercles which are undergoing caseation, others which would be considered as recently developed, and areas of pneumonic infiltration and confluent tubercles which appear to be regressing. The extent or total mass of tuberculous lesions in the lungs is considerably reduced during the 2nd and 3rd months after inoculation, and since cavitation is relatively rare at this period it would seem likely that many of the lesions regress in a manner entirely similar to that after the injection of dead organisms. It is probable that certain parenchymal infiltrations of tuberculosis, in infants, which resolve slowly, leaving but a trace of their existence roentgenologically, undergo a similar change as result of a like process.

SUMMARY

1. Rabbits vaccinated with tubercle bacilli killed by exposure to formalin (0.4 per cent) did not show any acquired resistance to subsequent infection with bovine tubercle bacilli, while rabbits vaccinated with tubercle bacilli which had been killed by heating to 70° for 1 hour survived more than half as long again as their controls.
2. Intraperitoneal injection of either the formalin-killed vaccine or the heat-killed vaccine into guinea pigs made them skin-sensitive to tuberculoprotein MA-100.
3. The rate of absorption of the formalin-killed vaccine when introduced beneath the the skin was similar to that of the heat-killed vaccine.
4. Following the intravenous injection of heat-killed tubercle bacilli, it was found that rabbits developed a massive tubercular pneumonia. A study of the production and ultimate absorption of the cellular exudate showed that these processes were similar to those found after the

injection of living bacilli. The lesions which followed the injection of heat-killed bacilli differed from the lesions found in active tuberculosis in that in any one animal they showed a striking uniformity in appearance, while in the active disease the lungs showed a great diversity in type of lesion. Studies of the blood cells during the period of injection of dead organisms showed that the changes which are characteristic of the period during which a tuberculous pneumonia develops in rabbits (30 to 40 days after inoculation) were faithfully reproduced. It is suggested that the process of regression described may be similar to that which occurs in childhood tuberculosis, in which rather extensive pulmonary lesions resolve without leaving evidence of damage to the parenchyma of the lung.

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A STUDY OF THE THERAPEUTIC MECHANISM OF ANTIPNEUMOCOCCIC SERUM ON THE EXPERIMENTAL DERMAL PNEUMOCOCCUS INFECTION IN RABBITS

III. The Influence of Non-Specific Factors

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In previous communications (1), it was observed that when large groups of rabbits are infected with a uniformly fatal dose of pneumococci and treated with type-specific antiserum, they do not all respond alike. It was found that, under standard conditions, with certain amounts of serum which are ineffective for the majority, some rabbits survive; on the other hand, certain larger doses of serum which are effective for the majority of rabbits, frequently fail to prevent the fatal termination of the infection in others. The factors which determine the death of some and the survival of others among a group of animals which are infected and treated in exactly the same manner, are not clear. Upon a proper understanding of these factors depends the true conception not only of the therapeutic mechanism of the action of specific antisera but also of the character of natural resistance to infection. The purpose of the present communication is to present experiments which deal with the effect of non-specific agents in modifying the response of pneumococcus-infected rabbits to treatment with specific antiserum.

It has been observed in previous experiments (1, 2), that a therapeutically effective antipneumococcic serum may have a certain so called subeffective dose which can yield roughly reproducible results. The subeffective dose employed in the earlier work was 0.3 cc. (containing 300 mouse protective units) of a Type I serum, which was administered 6 hours after intracutaneous infection with a uniformly fatal quantity of pneumococcus culture. Approximately 70 per cent of the rabbits treated with this amount of serum invariably died, whereas a larger dose of the same serum was capable of effecting a survival of 70 to 100 per cent. The study of the manner in which a subeffective dose differs from an effective one, and of the factors which might convert the former into the latter, therefore appeared capable of contributing to present knowledge of the curative action of antipneumococcic serum. It was found that one way in which this subeffective dose could be rendered effective, was by the addition of the so called non-antibacterial fraction of the serum, and that the extrinsic part of the immune mechanism was concerned also with substances other than the type-specific antibacterial bodies. It has long been maintained, however, that successful treatment of pneumococcus infection is dependent as much upon certain factors in the host as upon the substances which are supplied by the specific serum. In the present study an attempt was made to determine whether or not the effectiveness of the host factors might be increased by non-specific agents. Certain gold compounds, the influence of which to combat infection was being studied by one of us at the time, were chosen empirically for these experiments. Their therapeutic effect on the dermal pneumococcus infection in rabbits was studied both alone and in combination with the subeffective dose of Type I serum described above.

Method

Adult rabbits weighing approximately 1800 to 2400 gm. were used in these experiments; they were injected intracutaneously with 0.001 cc. of a fully virulent, 18 hour, standardized, Type I pneumococcus broth culture; the details of the procedure were described in the first communication. The various preparations were administered intravenously 6 hours after infection, when the bacteremia is already established and the local lesion is still very slight or even entirely absent. The gold compounds used were gold chloride, gold hexamethylenetetramine dissolved in sodium sulfite, and the latter combined with 0.007 per cent by weight of the cyclic fatty acids obtained from the saponification products of chaulmoogra oil (3); but, since only the quantity of gold present in the dose seemed to matter, the results will not be presented separately. In each experiment, one group of rabbits was treated with the gold compound only, one with the subeffective dose of serum only, and one with a combination of the two; there were usually not less than five and not more than ten rabbits in each group. The tests were repeated several times with rabbits obtained from different sources, and the results were practically the same each time. Daily rectal temperatures, dermal lesion measurements, and blood cultures (from the ear veins and arteries) were done for a period of 10 days as long as the rabbits lived. Necropsy was performed on each dead rabbit and cultures were taken from the heart's blood and from the brain. The results are summarized in Table I.

TABLE I

Influence of Gold Salts on the Response of Pneumococcus-Infected Rabbits to Treatment with a Subeffective Dose of Antiserum

Therapy	Number of rabbits used	Number survived	Survival
			per cent
None	40	0	0
Gold salts only, (Au 5 mg.) 7 rabbits—gold compound (*a*)* 10 " " " (*b*)†	17	1	6
Subeffective dose of serum only, 0.3 cc. Serum 624 Type I	31	9	29
Subeffective dose of serum and gold salts (Au 5 mg.) 12 rabbits—gold compound (*a*) 20 " " " (*b*) 20 " gold chloride	52	40	77
Subeffective dose of serum and gold salts (Au 2 mg.) 10 rabbits—gold chloride 10 " gold compound (*b*)	20	10	50
Subeffective dose of serum and gold salts (Au 1 mg.) 5 rabbits—gold chloride 5 " gold compound (*b*)	10	5	50

* Gold compound (*a*): gold hexamethylenetetramine dissolved in sodium sulfite and combined with 0.007 per cent by weight of the cyclic fatty acids obtained from the saponification products of chaulmoogra oil.

† Gold compound (*b*): gold hexamethylenetetramine dissolved in sodium sulfite.

RESULTS

Untreated Rabbits.—On various occasions, simultaneously with these tests, 40 rabbits were injected intracutaneously with the same amount of pneumococcus culture, but received no treatment whatever. There was not a single survival among them, death occurring with a progressively increasing bacteremia and local lesion, usually on the 3rd to 5th day after infection.

Rabbits Treated with Gold Compounds Only.—The various compounds (5 mg. of gold in one dose) were administered intravenously 6 hours after infection. Of the 17 rabbits thus treated, 16 died. No difference was observed in the course of either the systemic or local infection in this group as compared with the untreated rabbits. The one surviving rabbit recovered by crisis on the 7th day, after 6 days of a continued fever of 106°F. or more (107.4°F. on one occasion), with a spontaneous sterilization of a rather severe bacteremia on the 5th day. Except in the case of this one rabbit, the gold compounds alone appeared to exert no appreciable effect on the course and outcome of this experimental pneumococcus infection.

Effect of Subeffective Dose of Serum.—The effect of the subeffective dose of serum (0.3 cc. Type I serum, containing 300 mouse protective units) has already been described in the previous communications. This amount of serum, given 6 hours after infection, has practically no effect on the development of the dermal lesion in the majority of rabbits. There is at first a sterilization of the blood stream in all; in slightly more than half, the bacteremia recurs to a greater or lesser extent. Of the 31 rabbits treated with this dose of serum only nine or approximately 29 per cent survived. Only about half of those that died had either a persistent or terminal bacteremia. In the remainder, careful cultures of the heart's blood and of the brain, frequently obtained immediately after death, were entirely negative. In all of these rabbits the temperature usually remained elevated until the end, and death appeared to be due to absorption of toxins from the dermal lesions, which were very marked. Thus by imparting a partial immunity only, the pneumococcus infection in these rabbits becomes localized in the skin; and the subsequent course is one which closely simulates certain cases of pneumococcus pneumonia in man, in which there is no bacteremia, death apparently being the result of absorption of toxins from the local lesion.

Effect of Subeffective Dose of Serum Administered in Conjunction with Gold Compounds.—In these tests the serum and gold compounds were administered intravenously from separate syringes, 6 hours after infection; no other treatment was given. 52 rabbits received 5 mg.

of gold each, 20 rabbits 2 mg. of gold each, and 10 rabbits 1 mg. of gold each. No significant difference was observed among the groups treated with the various gold compounds; the results seemed to depend chiefly on the amount of gold in each dose. The most striking effect of this combined therapy proved to be the complete reversal of the death-survival ratio; whereas the 5 mg. of gold alone had almost no effect, and treatment with the subeffective dose alone yielded approximately 71 per cent deaths and 29 per cent survivals, there were instead only 23 per cent deaths and 77 per cent survivals, when the two were administered together. These results were readily reproducible with different groups of rabbits tested on different occasions. It is to be noted that the smaller doses of gold were not quite as effective. There were a small number of rabbits in which the disease was aborted. In the majority of rabbits, however, the dermal lesion seemed to be even more marked than in the controls, there was perhaps less tendency for recurrence of bacteremia, and the temperature was rather irregular; yet, whether in spite of, or because of the more severe local response, the greater number survived. Though the addition of gold failed to terminate the disease rapidly, by crisis, it nevertheless seems to have influenced the resistance of a great many rabbits to such an extent that they were able to withstand an infection which otherwise would have proved fatal for them.

DISCUSSION

It is generally maintained, though not fully appreciated, that a consideration of the therapeutic mechanism of the so called antibacterial sera must concern itself not only with what one may call the extrinsic factors supplied by the serum, but also with certain at present ill defined intrinsic host factors. That these intrinsic host factors may exhibit appreciable variation becomes apparent in the case of the experimental dermal pneumococcus infection in rabbits, when under standard conditions of infection and therapy, some die and others survive. In previous communications the rôle of some of the serum factors was analyzed and studied. The purpose of the present investigation was to determine whether or not non-specific agents were capable of exerting any influence on the response of the animal to serum therapy. The non-specific agent, empirically chosen, consisted of gold administered in the form of various compounds. It has been demonstrated in these experiments that whereas gold by itself had very little effect either on the course or the outcome of the experimental dermal pneumococcus infection in rabbits, it is nevertheless capable of exerting a definite and marked beneficial effect in animals treated with a subeffective dose of the specific antiserum. Of the rabbits treated with the subeffective dose of serum alone, 71 per cent died and only 29 per cent survived; the additional administration of gold reversed this death-survival ratio with the result that of a large group of rabbits which received the combined therapy, 77 per cent survived and only 23 per cent died.

The mechanism responsible for the difference described, is obscure, and an analysis of the modified course of infection aids but little in its elucidation. It is interesting to note, however, that in the majority of instances the disease was not precipitously aborted or terminated; but, while the local lesion was more severe than among the controls, there was less tendency for reinfection of the blood stream, and the larger number of rabbits survived with ultimate resolution of the dermal lesion. It might be suggested that the effect of the combined therapy was to localize the infection more thoroughly, which would account for the severer dermal lesions and the lower incidence of bacteremia; but although there may be some truth in such an assumption it fails to explain why these rabbits survived. It has been previously shown that among the rabbits treated with the subeffective dose of serum alone, there are a large number which die apparently as a result of an absorption of toxins from the local lesion; therefore, in addition to the increased localization of the infection one would have to assume the operation of still another mechanism which either inhibits absorption or aids the host in the detoxication of the absorbed products.

It is not unlikely that various other substances may be capable of exerting the influence exhibited by gold in these experiments. In view of the fact that gold is a well known "capillary poison" it is interesting to compare the effective amount used in this work with the lethal dose. Heubner (4) demonstrated that 7 mg. of gold per kilo is toxic for rabbits. The 5 mg. dose given to rabbits weighing 1800 to 2400 gm. is about one-third the lethal dose. Heubner further showed that the toxic amount of gold for Carnivora is three times greater than that which is toxic for rabbits, and that a lethal quantity of gold has no toxic action when given in divided doses over a period of a few hours.

In 1915, Moore (5) demonstrated that "a single small dose of ethylhydrocuprein (optochin base), which by itself has practically no protective effect against experimental pneumococcal infection in mice, is capable of increasing the threshold value of the type homologous antipneumococcus serum at least fifty times." Moore's experiments were protective in nature, the organisms having been mixed with the serum before injection, and the ethylhydrocupreine having been administered at the time of infection; the experiments reported in the present communication are curative not protective in character, the various agents having been injected after the infection had already become established. It is plain that, in protective as well as curative experiments, non-specific agents may greatly modify the response of pneumococcus-infected animals to treatment with type-specific antiserum. Further work in this direction may perhaps yield results which could be applied in the treatment of pneumococcus infection in man.

SUMMARY

The purpose of the present investigation was to determine whether or not non-specific agents were capable of exerting any influence on the response of pneumococcus-infected animals to specific serum therapy. It has been demonstrated in these experiments that whereas gold (empirically chosen) by itself had very little effect either on the course or the outcome of the experimental pneumococcus infection, it is nevertheless capable of exerting a definite and marked beneficial effect in rabbits treated with a subeffective dose of the specific antiserum. Of the rabbits treated with the subeffective dose of serum alone, 71 per cent died and only 29 per cent survived; the additional administration of gold reversed this death-survival ratio with the result that of a large group of rabbits which received the combined therapy, 77 per cent survived and only 23 per cent died.

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CHANGES IN THE TITER OF ANTIPNEUMOCOCCAL HUMORAL IMMUNITY IN ADULT HUMAN BEINGS

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Adult human beings, as a group, possess a relatively high degree of humoral immunity against the pneumococcus. This property of blood is a specific one, since it has been shown that individuals may vary greatly in their pneumococcidal activity against the different types (1–4). Little is known, however, concerning the constancy of this reaction of the blood of individuals of any particular age group. Ward[1] (2), who has made a few observations on this point, states that he noted no change in the titer of humoral immune substances in nine normal persons on whom pneumococcidal tests were repeated. No details were given in these instances as to the actual time interval elapsing between tests. That changes in the titer of the immune substances do occur under normal circumstances is suggested by the studies of Sutliff and Finland (4), who found that the incidence of pneumococcidal power in human beings varies with age. In rabbits Woo (5) has shown that the titer of antipneumococcal substances increases with the age of the animal.

The present investigation was undertaken to study the relative constancy of the degree of humoral immunity found in the individuals of the adult age group.

Materials and Methods

Pneumococcidal Tests.—These were carried out according to the technique described by Robertson and his coworkers (6, 7), employing quantitated mixtures of serum, washed leucocytes, and pneumococci in sealed tubes, which were agitated

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[1] Ward (2) retested one individual, from whom 40 to 50 cc. of blood had been withdrawn at frequent intervals over a period of 2 to 3 months, and found a diminution in amount of humoral immunity against Types I, II and III pneumococci.

during incubation. Human leucocytes were obtained from Group I (Jansky) donors.

The organisms used in the tests were laboratory strains of Type I and Type II pneumococci which had been kept at maximum mouse virulence by frequent animal passage since originally isolated. Preceding each group of tests animal passage was repeated. 0.1 cc. of a 10^{-7} dilution of a broth culture of each organism killed mice in repeated tests in from 24 to 36 hours.

Five dilutions of a standard suspension of these organisms (10^{-3} to 10^{-7} dilutions of a suspension containing approximately one billion pairs of pneumococci) were added to the tubes containing the mixture of leucocytes and test serum. The actual number of organisms in each dilution was estimated from the colony count of plates seeded with 0.1 cc. of the 10^{-7} dilution (theoretically to contain 100 organisms). Actually, the number of organisms found varied between 21 and 200 per 0.1 cc. of the suspension. This divergence of colony counts is taken into consideration in the following manner.

The counts in the 10^{-7} dilution of the Type I series varied between 75 and 200, and in the Type II series between 13 and 220. It so happened that all of the Type II series with colony counts below 68 showed pneumococcidal activity in the tube containing the 10^{-7} dilution. For this reason all cases in both Type I and Type II series could conveniently be grouped within the range of colony counts of 68 to 220, which would thus include the range of colony counts in the Type I series. The difference between the lowest and highest colony counts in the 10^{-7} dilutions in which growth occurred in the two series is thus equivalent to a 3.2 dilution.

It has been found that the pneumococcidal tests as carried out in this study are accurate only within one 10-fold dilution. Consequently, a difference in pneumococcidal power in repeated tests equivalent to a single 10-fold dilution is disregarded as within the limits of error of the method. To facilitate comparison of the tests with divergent colony counts a 3-fold dilution is also disregarded. Consequently, to exceed what we have thus established as experimental variation, there must occur a difference in pneumococcidal power, expressed in terms of the number of organisms killed, equivalent to a 300-fold dilution.

Cases Selected for Study[2]

Thirty-four individuals, 22 to 40 years of age, in good health, who were employed in various capacities in the Albert Merritt Billings Hospital, were first tested in 1931 in the latter half of the month of September and throughout the month of October. During this time nine members of the group of thirty-four developed a common cold, and serum specimens obtained at the time of maximum symptoms

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[2] Four of these cases gave a history of pneumonia 5 or more years previous to the time of this study. None of them had had pneumonia within the last 5 years.

were retested for comparison with their pneumococcidal power during the normal period. All of the group of thirty-four individuals were retested during March and April of 1932.

Twelve cases of a severe influenza-like infection occurring in epidemic form among the students of the University of Chicago were chosen for another series. Their ages varied between 20 and 35 years. The clinical course of this infection was similar in all cases. There were 3 to 4 days of temperature ranging from 100–104°F., marked malaise, generalized aching of muscles, mild sore throat, headache, and often a harsh, unproductive cough. There were no abnormal physical signs in the chest. The white blood cell counts ranged between 3,900 and 6,800. Blood samples for the pneumococcidal tests were obtained in most instances at the time of the highest temperature, or a day or so thereafter. The first series of determinations were made in February, 1932. 6 to 8 weeks later, after complete convalescence, a second blood sample was similarly tested. All pneumococcidal determinations were made within 1 to 2 days after procuring the blood samples.

Changes in Humoral Immunity in Normal Individuals

The results of the pneumococcidal tests of the thirty-four normal individuals are given in Table I. It is seen that about one-third of the individuals possessed no humoral immunity against the Type I pneumococcus, while only one case lacked this property against the Type II. A majority of the members of the series showed relatively high immunity against Pneumococcus Type II. With a similar technique Sutliff and Finland (4) and Ward (2) have demonstrated this same relative difference in pneumococcidal activity against these two types.

Considering a change of 300-fold dilution as significant in evaluating this data we found seven individuals who showed a decrease in pneumococcidal activity against Type I pneumococcus. Only two cases of diminished humoral immunity occurred against Type II. No increase in pneumococcidal power was found in this series. Of the seven individuals showing decreased pneumococcidal power toward Type I, two also had a decrease in activity toward Type II. The other five exhibited no significant change in their reaction to the Type

TABLE I

Pneumococcidal Promoting Activity of the Serum of Normal Individuals

Individuals	Type I pneumococcus No. killed Fall, 1931	Type I pneumococcus No. killed Spring, 1932	Change in titer of serum immune substances	Type II pneumococcus No. killed Fall, 1931	Type II pneumococcus No. killed Spring, 1932	Change in titer of serum immune substances
1. Po	0	0	0	220,000	28,000	0
2. Ed	0	0	0	144,000	44,000	0
3. Wa	0	0	0	14,400	52,000	0
4. Ma	0	0	0	70,000	5,200	0
5. Sm	0	0	0	7,000	28,000	0
6. Ca	0	0	0	74,000	52,000	0
7. Sa	0	0	0	680	186	0
8. Ha	0	0	0	680,000	130,000	0
9. Or	0	0	0	0	0	0
10. Re	0	0	0	220	1,860	0
11. Ke	200	0	0	23,000	28,000	0
12. Ch	91	0	0	74,000	13,000	0
13. Ro	126	0	0	68,000	130,000	0
14. Hi	1,530	0	Dim.	140,000	540	Dim.
15. Fr	1,530	680	0	1,400	520	0
16. Gi	1,530	680	0	140,000	52,000	0
17. Ri	19,800	0	Dim.	1,440,000	130,000	0
18. Da	19,800	8,100	0	1,440,000	44,000	0
19. He	19,800	8,100	0	144,000	28,000	0
20. Pa	126,000	0	Dim.	68,000	44,000	0
21. Se	126,000	0	Dim.	68,000	44,000	0
22. Sa	15,300	14,500	0	140,000	186,000	0
23. Ha	15,300	80,000	0	140,000	280,000	0
24. Ha	20,000	76,000	0	7,000	130,000	0
25. Br	91,000	81,000	0	74,000	44,000	0
26. St	1,980,000	6,800	Dim.	1,440,000	520,000	0
27. Ro	78,000	8,000	0	7,400	2,800	0
28. Ki	2,000,000	8,000	Dim.	70	0	0
29. J. H.	1,980,000	760,000	0	1,440,000	130,000	0
30. Re	15,300	80,000	0	14,000	54,000	0
31. Fr	91,000	81,000	0	74,000	44,000	0
32. Ma	1,260,000	800,000	0	680,000	28,000	0
33. V. S.	15,300	0	Dim.	1,400	0	Dim.
34. M. H.	780,000	680,000	0	7,400	5,200	0

In the tables, dim. indicates diminished; incr., increased.

II pneumococcus. The majority of cases showed no particular change in their titer of immune substances in the period between the two series of tests. It is to be noted that the number of microorganisms killed in the spring tests in individual instances tended to be less than in the fall, although the difference did not exceed the 300-fold dilution. One possible reason for this may have been that the colony counts in the respective dilutions happened to be lower in the spring tests.

Examination of the data in the Type I series on the basis of the dilution at which growth occurred rather than the number of microorganisms killed gives the same contrast between the two series of tests. No change occurred in twenty-eight cases. Four showed a drop in titer of three dilutions, three of two dilutions, and five of one dilution. Three cases showed an increase in pneumococcidal activity equivalent to one dilution.

Another point to note is the fact that decrease in titer of pneumococcidal power for one type was not necessarily associated with a change in reaction toward the other type.

Effect of Colds

In the series of nine individuals tested while suffering from colds, five exhibited a definite change in pneumococcidal power of their blood (Table II). One showed a decrease against Type I but no alteration in reaction toward Type II. Of the four cases with altered reaction toward Type II pneumococci, two showed an increase, and two a decrease. In none of these instances was there any change in their reaction toward the other organism.

At least fourteen of the thirty-four normal persons mentioned above had colds in the interval between the two sets of pneumococcidal tests. Three of these were among those showing diminution in pneumococcidal titer. Three other cases in the normal series with a similar alteration in their degree of immunity were free of colds throughout the period covered by these tests.

There was thus no uniform response to colds, and we are inclined to interpret these findings as having no relation to the cold itself but to represent fluctuations due to some other cause. It is seen, for instance, in Cases 5, 6, and 8, that the change was a gradual one and not an abrupt reaction to the cold.[3]

[3] The first two tests in eight of the cases were made at intervals of 1 to 3 weeks. In the ninth case 7 weeks elapsed between the normal test and the "cold" test. The last determinations were made from 5 to 6 months after the initial ones.

TABLE II

Pneumococcidal Promoting Activity of the Serum of Subjects Having Colds

Individuals	Type I pneumococcus			Change in titer of serum immune substances	Type II pneumococcus			Change in titer of serum immune substances
	No. killed				No. killed			
	Normal	Cold	Normal		Normal	Cold	Normal	
	Fall, 1931	Fall, 1931	Spring, 1932		Fall, 1931	Fall, 1931	Spring, 1932	
1. Fe	1,980	0	76	Dim.	1,440	2,200	13,000	0
2. Gr	780,000	2,000,000	81,000	0	74,000	7,000	44,000	0
3. Sc	200	0	0	0	70,000	25,000	54,000	0
4. St	168	0	0	0	0	2,500	4,400	Inc.
5. Co	0	0*	0	0	2,500	70,000*	280	Dim.
6. Mi	0	0	0	0	220,000	25,000	186	Dim.
7. Co	780,000	1,740,000	680,000	0	74,000	2,200,000	520,000	0
8. Ei	9,100	1,740	1,300	0	740	20,000	520,000	Inc.

* In this case the test during the cold preceded the first test when patient was normal.

TABLE III

Pneumococcidal Promoting Activity of the Serum of Patients with Influenza-Like Infection

Individuals	Type I pneumococcus		Change in titer of serum immune substances	Type II pneumococcus		Change in titer of serum immune substances
	No. killed			No. killed		
	Influenza-like infection*	Normal*		Influenza-like-infection*	Normal*	
1. Ha	0	0	0	0	5,400	Inc.
2. Sc	0	0	0	0	0	0
3. Cl	0	0	0	4,900	5,200	0
4. Be	0	0	0	6,300	1,860	0
5. Bu	0	80	0	4,900	54,000	0
6. Sh	0	0	0	63,000	54,000	0
7. Jo	97,000	80	Dim.	0	0	0
8. Mo	97,000	130,000	0	4,900	52,000	0
9. As	0	0	0	4,900	18,600	0
10. Re	600	0	Dim.	6,300	52,000	0
11. Ba	600,000	1,450,000	0	6,300	18,600	0
12. Fl	60,000	14,500	0	630,000	18,600	0

* 6 to 8 weeks elapsed between the two series of tests.

Effect of Influenza-Like Infection

Nine of the twelve cases tested showed no change in the pneumococcidal promoting power of their serum (Table III). In two cases (Nos. 7 and 10) there was a decrease in this serum property forPneumococcus Type I, and in one (Case 1) an increase for Type II. These tests were done about 2 months apart in the spring of 1932. Here again we note no uniform change in titer as a result of this influenza-like upper respiratory infection.

DISCUSSION

There are certain limitations to the use of pneumococcidal tests for this type of study, although the method is ideally adapted for measuring the relative pneumococcidal activity of different blood samples when tested simultaneously. In such experiments the serum-leucocyte mixtures, the state of the microorganism, and the dilutions of the standard pneumococcus suspension are identical for each specimen. In the present experiments the tests in the several serum samples of each individual were done at different times which may have introduced variations in the constituents of the test. The most likely variable would seem to be the possible occurrence of changes in the virulence of the pneumococcus used. While pathogenicity for animals was maintained by frequent passage, we cannot be certain that maximum virulence for human beings is preserved by this means. Thus the possibility cannot be dismissed that some, if not all, of the changes noted in the pneumococcidal activity of adult serum were due to biological variations in the pneumococci used in the tests. However, if this were the only explanation of the observed differences we should have expected a uniform reaction of all individuals to this one factor. Actually, the majority of individuals showed no change in the degree of the pneumococcidal promoting activity of their serum. On several of the persons in this series we have data from pneumococcidal tests done 1 to 2 years previously with precisely the same technique and the same strains of Type I and Type II pneumococci. One individual (No. 23. Ha, Table I), tested in 1929, had no Type I pneumococcidal substances in her blood, although in 1931 she had a high degree of immunity against this organism. This particular person had been working in the laboratory with Type I pneumococci during these intervening years. In the same period no change in pneumococcidal activity toward Pneumococcus Type II had occurred. Three other individuals tested in 1930 with the same strains have shown no change in their degree of immunity toward these organisms. The data presented in this paper is insufficient to permit any detailed analysis of the possible factors concerned in the changes which we have noted. One might suspect from the studies here reported that seasonal variation was a factor. The tests done in the fall of 1931 were made on individuals in good health who had had the usual advantages of summer outdoor activities. These same subjects in the subsequent months were closely confined indoors during the daylight hours by their duties about the hospital. However, an adequate evaluation of this or any other factor would involve a protracted study over a period of years of a large group of people.

SUMMARY

Fifty-five individuals were tested to determine the pneumococcidal promoting activity of their serum against Types I and II pneumococci. By repeated tests an attempt was made to study the constancy of the degree of their immunity over intervals of 2 to 6 months. In this group were included nine persons with common colds and twelve cases of a severe influenza-like infection. Fifteen of the fifty-five cases showed a change in titer of their humoral immunity against either Type I or Type II or both. Three of these showed an increase, and twelve a decrease. This reaction in most instances was a specific one in that the altered reaction toward one type was not associated with a similar change toward the other type pneumococcus. Colds and influenza-like infections apparently exerted no effect upon the titer of humoral immune substances.

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LYMPHOMATOSIS, MYELOMATOSIS, AND ENDOTHELIOMA OF CHICKENS CAUSED BY A FILTERABLE AGENT*

I. TRANSMISSION EXPERIMENTS

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Plates 14 and 15

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The filterable agent that will be described here produces in chickens leukosis of types that hitherto have not been successfully transmitted, namely lymphomatosis and myelomatosis, sometimes with leukemia and sometimes with tumor formation. In many chickens inoculated with this strain leukosis was associated with the formation of tumors that histologically resembled endothelioma. Infiltration of nerves often occurred among chickens inoculated with this strain, and in a few birds paresis or paralysis of legs or wings was noted. Thus this transmissible strain possesses characteristics ascribed to three different types of filterable agents; namely, (*a*) the agent of Ellermann and Bang (1) that causes leukosis of chickens; (*b*) the agents of Rous that stimulate connective tissues of chickens to neoplastic growth and may cause endothelioma (Begg (2)); and (*c*) the agent of Pappenheimer, Dunn, and Cone (3) that produces fowl paralysis.

In this article evidence will be presented to show that lymphomatosis, myelomatosis, endothelioma, and fowl paralysis occurring among the passages of this strain are caused by filterable agents. In the paper that follows, the relationship of these diseases and the anatomical lesions associated with them will be discussed. The available evidence is in favor of the assumption that a single agent can produce lymphomatosis, with or without paralysis, myelomatosis, and endothelioma.

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Nomenclature

Leukosis will be used as a collective term to designate diseases characterized by autonomous growth of the cells of the blood-forming organs of chickens. Leukosis is subdivided into lymphoid, myeloid, and erythroleukosis, according to the three types of blood-forming systems which may become affected. The adjectives leukemic, subleukemic, and aleukemic are used to indicate the degree of blood involvement.

In erythroleukosis the primitive erythroblasts multiply rapidly in the bone marrow (4), and, without maturing, enter the circulation in large numbers and accumulate in the pulp of the spleen and the capillaries of organs such as lungs and liver.

Myeloid leukosis occurs in two distinct forms: myeloblastomatosis and myelocytomatosis. Myeloblastomatosis is characterized by the appearance of enormous numbers of large mononuclear cells, presumably myeloblasts (Ellermann), in the blood. This type of leukosis is not associated with tumors and is caused by the same agent that causes erythroleukosis (5). In myelocytomatosis, myelocytes are numerous in the blood, whereas myeloblasts are few or absent. This form is usually associated with tumor formation. The differentiation between myeloblastomatosis and myelocytomatosis becomes unavoidable in the study of transmissible leukosis because the agent of the common type of transmissible leukosis (5, 6) produces erythroleukosis and myeloblastomatosis almost exclusively, while the strain to be described here produces lymphomatosis and myelocytomatosis. Mathews (7) has noted the difference between myeloid leukemia of Ellermann and myelocytomatosis, and described the latter disease under the name of "leukochloroma."

Lymphomatosis (lymphoid leukosis) of chickens is identical with the similar disease of mammals. It has been described recently by Mathews and Walkey (8).

Endothelioma presents great variation in location and in the histological characteristics of its lesions. It may occur as a solid tumor composed of large round cells, of which several are binucleated, or as an angiomatous growth with extensive hemorrhages, or, most commonly, as a tumor consisting of pleomorphic cells with numerous large necrotic areas surrounded by multinucleated giant cells. The nuclei of all these cells are similar: they are large and spherical, contain little chromatin (indicated by their pale color in hematoxylin-eosin-stained preparations), and have a large nucleolus.

Transmissible Strains.—The common type of transmissible strain described in previous reports (5) will be referred to as Strain 1, the strain described here as Strain 2.

LITERATURE

1. Chicken Leukosis.—Ellermann and Bang have stated that all types of avian leukosis are caused by one filterable virus, but the evidence presented by them is only sufficient to show that two types of leukosis, namely erythro- and myeloid leukosis, are transmissible and are caused by the same filterable agent (5, 6). Andersen and Bang (9) failed, in numerous attempts, to transmit lymphatic leukosis, and Bang (10) expressed recently the view that lymphatic leukosis had so far not been successfully transmitted. Erythroleukosis and myeloid leukosis, on the other hand, are readily transmissible. Furth (5), Engelbreth-Holm (6) Stubbs and Furth (11), and Olson (12) have described transmissible strains that produced erythroleukosis and myeloid leukosis. Jármai (13) described a strain that produced only erythroleukosis. Battaglia and Leinati (14) observed a strain that produced erythroleukosis, myeloid leukemia, and "hemocytoblastic myelosis" (identical with Ellermann's lymphoid leukosis).

2. *Fowl Paralysis.*—Van der Walle and Winkler-Junius (15) were the first to suggest, though on insufficient evidence, that fowl paralysis is caused by a filterable virus. The extensive investigations of Pappenheimer, Dunn, and Cone (3) have shown that fowl paralysis is transmissible. About 25 per cent of the chickens inoculated by them developed paralysis, whereas in uninoculated control chickens the incidence of this disease was about 7 per cent. The anatomical lesion leading to paralysis is, according to these investigators, in some instances an inflammatory process (edema, accumulation of lymphocytes, plasma cells, and monocytes) and in other instances, a neoplastic process (lymphoma or lymphosarcoma). They observed lymphomatous infiltrations of viscera in about 10 per cent of the paralyzed chickens and presented suggestive evidence that transmission of fowl paralysis is due to a filterable virus. Further evidence for the causation of fowl paralysis by a filterable agent was presented by Seifried (16), while the data presented by Biely, Palmer, and Asmundson (17) strengthen the evidence that the lymphoid infiltration in the nervous system and in the viscera may occur in response to a common agent.

3. *Rous Sarcoma.*—Begg (2) describes a strain of chicken sarcoma in which the predominating type of cell resembles endothelium. The filterable agent of this tumor causes accumulation of lymphocytes and macrophages, followed by a tumor-like growth of endothelium of blood vessels with metastases in distant organs. The agent of Begg's endothelioma does not affect the blood-forming system and is immunologically related to other types of Rous sarcoma (Andrewes (18)). Roffo's round cell sarcoma (19) is possibly another link between chicken leukosis and Rous sarcoma.

Notes on the Presentation of Experimental Data; Difficulties in Their Interpretation

The figures shown in the tables refer to inoculations made before Dec. 31, 1932. An occasional observation made since will be mentioned in the text. Birds dying of intercurrent diseases from 1 to 2 months after inoculation are listed in separate columns, those dying two months after inoculation without showing any evidence of leukemia are given among the unsuccessful inoculations.

The disturbances in which the leukocyte count exceeded 100,000 and immature leukocytes formed the majority of the cells are called "leukemic." Those in which the leukocyte count was below 100,000 and sufficient immature leukocytes were present in the blood to make the type of leukosis recognizable are called "subleukemic," and the rest are called "aleukemic."

Instances in which the disturbance of one system was distinctly predominant are classified as myelomatosis, lymphomatosis, or erythroleukosis, those in which the involvement of two or more systems was conspicuous are given as "atypical" leukosis.

The incubation period of leukosis is variable; most of the chickens developed leukosis from 2 weeks to 2 months after inoculations, but in rare instances the incubation period was as long as 4 months. Thus our destruction of the animals after approximately 5 months is not an ideal procedure, but so far we have been able on account of limited space to keep only a few of the inoculated animals alive for longer periods.

Whereas in some instances it was extremely difficult to determine from postmortem appearances the success of inoculations and the type of leukosis, diagnosis could easily be made from blood smears. Basophile lymphocytes (Fig. 4), often containing minute vacuoles (Fig. 5), were present in the blood smears of most of the chickens successfully inoculated with this strain, even in cases in which the hyperplasia of the myeloid and erythroblastic tissues was predominant. Since such cells were absent in the blood smears of all control chickens, including those injected with Strain 1, their presence in the blood was characteristic of Strain 2 and distinguished the erythroleukosis and myeloid leukosis of Strain 2 from those of Strain 1.

Origin of the Transmissible Strain 2

This strain originated in a Barred Rock chicken (No. 2255) of about 8 months of age. The chicken was received November 19, 1931, weighing 670 gm., and was kept as an uninjected control among chickens inoculated with the transmissible Strain 1. This was done to find out whether leukosis is transmitted by natural means from diseased to healthy animals. There was no evidence that healthy animals acquire leukosis by contact with leukemic chickens and the type of lymphomatosis which developed in this chicken was different from that caused by any known transmissible strain. Therefore it must be regarded as a spontaneous disease.

Blood smears of this chicken taken from Nov. 20, 1931, to Feb. 27, 1932, were normal. The blood smear taken on Mar. 28 showed a few immature red cells. By Apr. 13, the abdomen of this chicken was distended by fluid, and the blood smear showed many polychrome erythrocytes and a few polychrome erythroblasts. On Apr. 23, immature lymphocytes were in the circulation in large numbers, the white cell count was 17,500, and 7 days later it was 130,000, most of the cells being large lymphocytes (Figs. 3, 4), many in mitosis. Post mortem the carcass weighed 1050 gm., the liver 100 gm., the spleen 7 gm. The liver was thickly studded with partly confluent tumor nodes (Figs. 2, 6) and similar tumor nodes were found in the spleen, heart, and kidneys (Figs. 1, 9). The size of these tumors varied from those just visible to those measuring 1 cm. across. They were composed of large round cells like lymphocytes, and other larger, paler stained cells which appeared to form a syncytial mass (Fig. 8). The bone marrow was reddish grey and contained minute, well defined, greyish, tumor-like areas.

Transmission Experiments

The results of the inoculations with blood and emulsion of tumor cells of this chicken are shown in Table I, and some of the salient data on the successfully inoculated chickens are shown in Table II.

Thus all birds successfully inoculated developed lymphomatosis, two aleukemic, three subleukemic, and one leukemic. One chicken (No. 2572) died 53 days after inoculation with severe anemia associated with osteosclerosis (osteodystrophia).

Text-fig. 1 shows the sequence of passages attempted subsequently from 66 chickens and more details of the first 37 passages are given in Table III.

Of the attempts to transfer leukosis from 53 chickens only eight were unsuccessful (Text-fig. 1).

Table III shows that about one-third of all inoculated chickens developed leukosis. More than one-half (72) of the successfully inoculated chickens developed lymphomatosis, and in a smaller number of chickens (38) several blood-forming systems (including the lymphatic system) were markedly affected. In 19 birds myelomatosis was predominant, and other blood-forming systems were affected slightly, if at all. In eight birds the alterations histologically resembled erythroleukosis. Endothelioma with giant cell formation was found in 11 birds. Instances of all these diseases will be described in the paper that follows. Myelomatosis appeared in the second passage, endothelioma with giant cell formation in the third passage, and both reappeared in all subsequent passages.

In the following section the results of the transmission experiments will be analyzed, and attempted transmissions with material contain-

TABLE I

The Results of Injections with Blood and Tumor Cells of Chicken 2255 (Spontaneous Leukemic Lymphomatosis)

	No. of birds injected	No. of successful transmissions	No. died of intercurrent disease
Intravenous injections with blood			
Chicks weighing from 150 to 350 gm.	20	1	8
Chickens weighing from 600 to 1000 gm.	9	4	1
Intramuscular and subcutaneous injections			
Chicks weighing from 230 to 290 gm.	6	1	5
Chickens weighing from 650 to 690 gm.	3	0	0

TABLE II

Data on Chickens Successfully Inoculated with Material from Chicken 2255 (Spontaneous Leukemic Lymphomatosis)

Chicken No.	Weight of bird	Material injected	Route of injection	Amount injected	Incubation period	Duration of illness	Length of life after inoculation	Type of leukosis
	gm.			*cc.*	*days*	*days*	*days*	
2573	1000	Blood	iv.	2	16	9	K* 25	*sL*
2643	650	"	"	4	21	23	K 44	*lL*
2644	625	"	"	14	—	—	D* 143	*sL*
2645	595	"	"	1	64	20	D 84	*sL*
2618	250	"	"	5	7	1	K 8	*aL*
2619	290	Tumor	im.	—	—	—	D 80	*aL*

* D = died, K = killed.

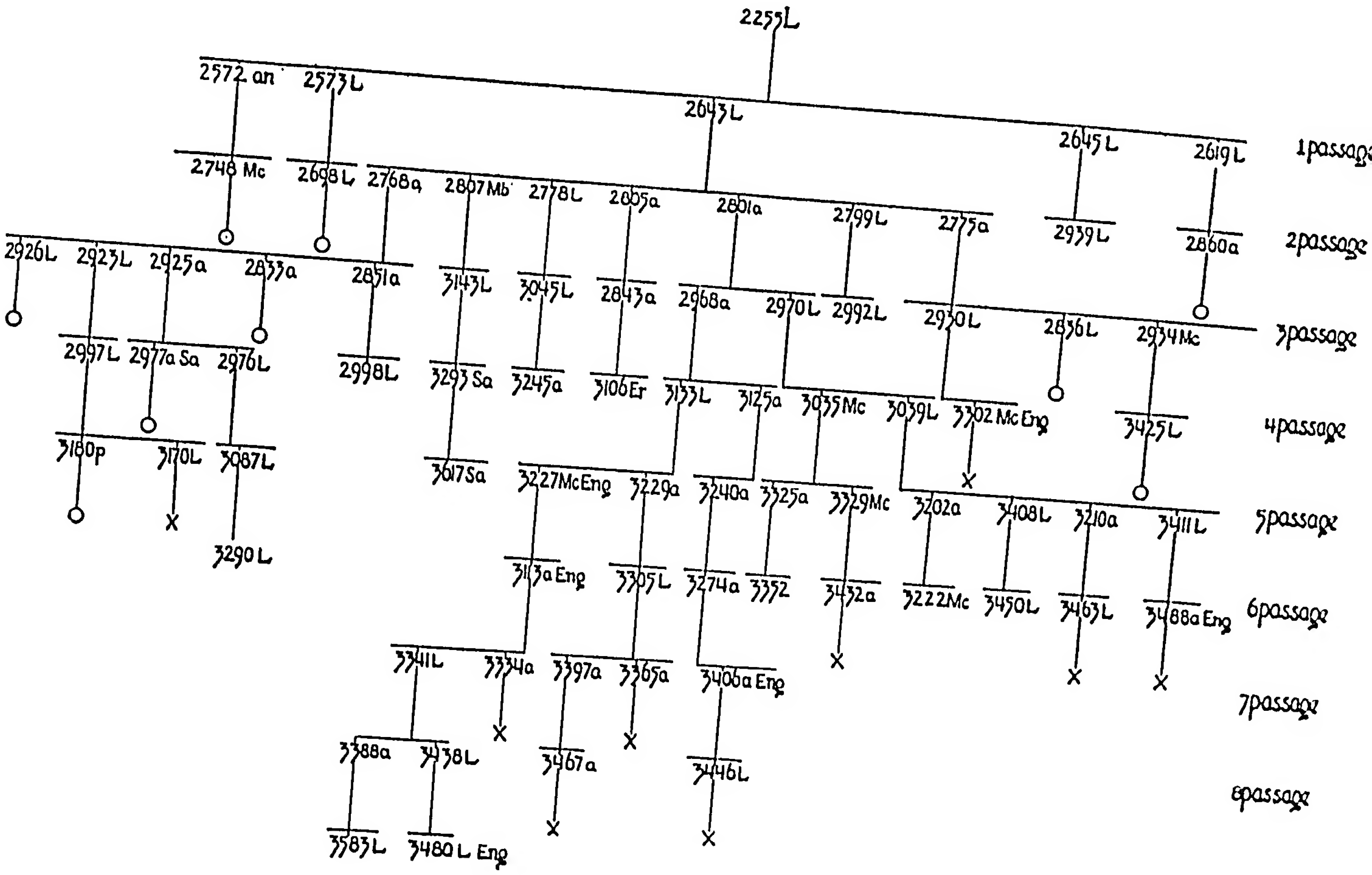
Abbreviations Used in Text-Fig. 1 and in All Tables

Types of Disease.—*L* = lymphoid leukosis; *Mb* = myeloblastomatosis; *Mc* = myelocytomatosis; *Er* = erythroleukosis; *En* = endothelioma; *Eng* = endothelioma with giant cell formation; *Sa* = sarcoma formed by cells other than round cells; *a* (used only in Text-fig. 1) = atypical (mixed) leukosis; *an* = anemia; *p* = paralysis (neurolymphomatosis) without *L* of viscera.

Degree of Blood Involvement.—*l* = leukemic; *s* = subleukemic; *a* (used only in the tables) = aleukemic.

Route of Injection.—iv. = intravenous; im. = intramuscular; sc. = subcutaneous; ip. = intraperitoneal.

o = unsuccessful transmission; *x* = transmission attempted recently.



TEXT-FIG. 1. Passages of Strain 2 of leukosis of fowls.

TABLE III

Transmission Experiments with Strain 2

Donor		No. of passages	Material injected	Route of injection	No. of transmissions			No. died of intercurrent disease		Type of leukosis					
No.	Type of leukosis				Successful	Uncertain	Unsuccessful	Within 1 mo.	From 1 to 2 mos.	Lymphomatosis	Myelocytomatosis	Myeloblastomatosis	Erythroleukosis	Atypical	Untyped
2255	*lL*	Spontaneous	Blood	iv.	6	1	13	7	2	5					1
			Tumor	sc. and im.	1	0	3	4	1	1					
2572	Anemia	I	Blood	iv.	2	0	3			1	1				
2573	*sL*	I	"	"	0	0	10	1	3						
			Tumor	im. and ip.	0	0	4		2						
			Plasma	iv.	1	0	3				1				
			Filtrate	"	0	0	4	1							
2618	*aL*	I	Blood	"	0	0	1	3							
2619	*aL*	I	"	"	1	0	7	1	1					1	
			Tumor	"	1	0	5			1					
			"	sc. and im.	0	0	4	1	2						
2643	*lL*	I	Blood	iv.	21	0	10	6	1	11	2	1		4	3
			Dried blood	"	0	0	4								
2645	*sL*	I	Blood	"	1	0	6		1	1					
2768	Atypical	II	"	"	13	1	4	2	2	6	1		2	3	1
			Glycerinated blood	"	0	0	3		2						

2778	aL	II	Blood	iv.	1	0	2	1			1				
			Tumor	"	3	0	1			2					1
2775	Atypical	II	Blood	"	4	0	7	1	1	2	1				1
			Plasma	"	2	0	3			2					
			Tumor	sc. and im.	2	0	8*		1						2
2709	sL	II	Blood	iv.	3	0	2			1				1	1
2801	Atypical	II	"	"	3	0	0			2				1	
			Tumor	"	1	0	2*			1					
2805	"	II	Blood	"	2	0	3	1	1					2†	
2860	Anemia	II	"	"	0	0	6								
2998	aL	II	Tumor	"	0	0	4		1						
2748	Mfs	II	Blood	"	0	0	5		1						
2807	Ma	II	"	"	4	0	3		1	3†				1	
			Plasma	"	4	0	3	1		1	1†			1	1
2844	Atypical	III	Blood	"	0	0	1	4							
2851	"	III	"	"	2	0	3			3					
3033	sL	III	"	"	2	1	1			3†					
3045	Atypical	III	"	"	5	0	0			4				2*	

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* Non-leukemic tumor.

† [illegible], with giant cell formation (one sign for each instance).

TABLE III—*Concluded*

Donor		No. of passages	Material injected	Route of injection	No. of transmissions			No. died of intercurrent disease		Type of leukosis					
No.	Type of leukosis				Successful	Uncertain	Unsuccessful	Within 1 mo.	From 1 to 2 mos.	Lymphomatosis	Myelocytomatosis	Myeloblastomatosis	Erythroleukosis	Atypical	Untyped
2926	*lL*	III	Blood	iv.	0	0	2		2						
			Plasma	"	1	0	3		2					1	
2970	*sL*	III	Blood	"	3	0	1			2	1				
			Plasma	"	1	1	2		1		1				
2836	*sL*	III	Blood	"	0	0	6								
2843	Atypical	III	"	"	4	0	1					1	2	1	
2968	"	III	"	"	4	0	8		1	2			1	1	
			Plasma	"	2	1	4			1			1		
3045	*lL*	III	Blood	"	2	0	2						1	1	
3143	*sL*	III	"	"	1	0	2*	1	1	1					
2930	*aL*	III	Tumor	"	1	0	4				1†				
2934	*sMc*	III	Blood	"	1	0	3			1					
2976	*sL*	IV	"	"	4	0	0			2*				1	1

2977	Atypical, *Sa*	IV	Tumor	im	0	0	4								
2997	*sL*	IV	Blood	iv.	2	1	5	1	2	1				1	
			Filtrate	"	3	0	3		3	3					
3039	*sL*	IV	Blood	"	5	0	0			2				3	
			Filtrate	"	2	0	6							2	
			Dried blood	"	4	0	1			4					
3125	Atypical	IV	Blood	"	3	0	1				1†			2	
3133	*sL*	IV	"	"	5	0	2	1		1†	2†			1	1
			Filtrate	"	0	0	6								
3035	*lMc*	IV	Blood	"	3	0	1				1			2†	
3202	Atypical	V	Filtrate	"	1	0	4		1				1		
3087	*sL*	V	Blood	"	2	0	2	1		2					
3240	Atypical	V	"	"	5	0	0			2				3	
3227	*aMc Eng*	V	"	"	3	0	2				2			1†	
3229	Atypical	V	"	"	3	0	2			1				2†	
Total........					150	6	212	38	36	72	17	2	8	38	13

ing immature, viable lymphocytes will be considered separately from attempted transmissions made with material free from viable cells.

All birds used in this study (including the controls) were Barred Rock chickens raised for us on one farm. For technique, see our previous communications (5).

Transmission of Strain 2 by Material Containing Viable Lymphocytes

Transmission of this strain was more often successful with blood than with an emulsion of tumor cells (Table IV). Inoculations with blood were successful both in chicks weighing from 100 to 300 gm. and in chickens weighing from 600 to 1200 gm. In the latter about one-half of all injected birds developed lymphomatosis, and the death rate due to intercurrent diseases was much lower than among baby chicks (Table IV). Transmission by an emulsion of tumor cells was much more successful by intravenous than by subcutaneous or intramuscular injections. In the seven experiments recorded in Table IV, only one of the 19 chickens injected intramuscularly died of leukosis.

TABLE IV

Inoculations with Material Containing Viable Lymphocytes

Material injected	Route of injection	No. of transmissions			No. died of intercurrent disease		Type of leukosis					
		Successful	Uncertain	Unsuccessful	Within 1 mo.	From 1 to 2 mos.	Lymphomatosis	Myelocytomatosis	Myeloblastomatosis	Erythroleukosis	Atypical	Untyped
Blood injected into chicks	iv.	15	1	31	16	6	3	2	1		5	4
Blood injected into young chickens	"	107	5	93	16	14	52	11	1	6	29	8
Tumor tissue	"	8	0	21		2	4	1				3
" "	im. and sc.	1	0	18	5	5	1					
Total		131	6	163	37	27	60	14	2	6	34	15

In more recent experiments not described fully in this report, subcutaneous and intramuscular inoculations were more often successful, but with one exception there was no tumor formation at the site of injection. On the other hand, subcutaneous transmissions of lymphomatosis of mice (20) were never successful unless a tumor developed at the site of inoculation and tumors could be produced with lymphocytes obtained from the blood. The significance of this observation will be discussed later.

TABLE V

Data on the Relationship between the Success of Inoculation and Amount of Blood Injected

Donor	No. of chicken injected	Amount of blood injected		Result of inoculation
		cc.		
2255, *lL*	2642	1	Killed after 152 days*	Negative
	2645	1	Died after 84 days	*sL*
	2643	4	Killed after 44 days	*lL*
	2641	10	Died after 132 days	Negative
	2644	14	" " 147 "	*lL*
2968, atypical	3160	0.5	Died after 40 days	*sL*
	3161	0.5	" " 62 "	*sL*
	3162	0.5	Alive and healthy	
	3163	10	" " "	
	3164	20	Died after 32 days	*E*
2643, *lL*	5 chicks	0.1	3 died of leukosis after 67, 75, and 123 days	
	5 "	1	3 " " " " 34, 75, " 116 "	
			(2 of each group are alive and healthy)	
	4 chickens	0.1	4 died of leukosis after 38, 49, 55, and 97 days	
	2 "	2	1 " " " " 41 days	
			(1 of the 2nd group is alive and healthy)	
3133, *sL*	4 chickens	0.005	3 died with leukosis after 40, 54, and 88 days	
	3 "	1	2 " " " " 51 and 86 days	
			(1 of each group is alive and healthy)	

* After injection.

The relationship between the success of inoculation and the amount of blood injected has thus far not been studied systematically. The observations shown in Table V suggest that the concentration of the agent in the blood is great and that the success of inoculation depends rather on the individual susceptibility of the chickens than on the inoculating dose.

Transmission Experiments with Material Free from Viable Cells

Table VI shows that all types of disease produced by material containing viable blood cells are also produced by material free from viable cells with the exception of myeloblastomatosis.

Transmission by Filtered and Unfiltered Plasma

Inoculations with unfiltered plasma were successful in about 36 per cent of the injected chickens, but after filtration through a Berkefeld N filter this percentage decreased to about 21.

The plasma was obtained by spinning heparinized blood at 1000 R.P.M. for 5 minutes, and then spinning the plasma twice at 3000 R.P.M., 10 minutes each time.

TABLE VI

Intravenous Inoculations with Material Free from Viable Leukocytes

Material injected	No. of transmissions			No. died of inter-current disease		Type of leukosis					
	Successful	Uncertain	Unsuccessful	Within 1 mo.	From 1 to 2 mos.	Lymphomatosis	Myelocytomatosis	Myeloblastomatosis	Erythroleukosis	Atypical	Untyped
Plasma, unfiltered	11	2	18	1	3	4	3		1	2	1
Plasma, passed through Berkefeld filter	6	0	23	1	4	3			1	2	
Dried blood	4	0	5			4					
Glycerinated blood	0	0	3	0	2						
Blood frozen at −70°C. for 30 min.	2	0	1							1	1
Total	23	2	50	2	9	11	3		2	5	2

After each centrifugalization, the plasma was pipetted off with great care to avoid contamination by blood cells. From 0.2 to 3 cc. of plasma was injected intravenously into each bird.

In filtration tests the plasma was first passed through a coarse Berkefeld filter, approximately 1 3/8 x 5/8 inches in size, and then refiltered through a medium or fine filter of the same size at a pressure of 40 cm. Hg. The filters were tested and washed with Locke's solution before filtration. Their quality is indicated by the following figures.

Plasma from chicken No.	1st filter		2nd filter	
	Bubbling pressure	Flow of water*	Bubbling pressure	Flow of water*
	cm.Hg	*cc.*	*cm.Hg*	*cc.*
2997	33	98	50	46
3039	29	78	46	46
3133	30	50	50	25
3202	22	50	50	52

* During 2 minutes at 40 cm. Hg.

From 0.2 to 5 cc. of filtrate was injected intravenously into each bird. For the results of the individual tests see Table III.

It is highly probable that the disease produced by unfiltered leukemic plasma was due to the cell-free agent that it contained and not to contaminating blood cells and that the smaller number of successful inoculations with filtered plasma was due to a partial retention of the agent by the filters.

In three more recent experiments plasma filtered through a medium filter caused leukosis in about two-thirds of the 22 inoculated chickens. These experiments will not be fully described because several of the chickens injected in this series are still alive.

Transmission Experiments with Dried Blood

The following experiment indicates that drying does not destroy the transmitting agent of Strain 2.

Procedure.—Loosely packed blood cells were frozen with the aid of solid carbon dioxide and dried in high vacuum over phosphorus pentoxide. In the desiccator the material to be dried rested on a metal box into which solid carbon dioxide was inserted through an outlet on the top of the desiccator. With this arrangement drying took place while the material was in the frozen state. The dried material was then kept in the ice box in sealed test-tubes.

Experiment 1.—40 cc. of blood was drawn from a chicken with subleukemic lymphomatosis (No. 3039) and the plasma, filtered through a Berkefeld N filter, was injected into eight chickens in amounts of 0.2 to 2 cc. Loosely packed cells from the same chicken were injected in amounts of 0.01 cc. into five chickens; cells 87 days after drying were injected in amounts corresponding to 1.2 cc. of loosely packed cells into another five; all injections were intravenous. Filtered plasma caused leukosis in only two of the eight injected chickens; one recovered and the other was killed 26 days after injection. Blood cells caused leukosis in all of the five injected chickens and they died 39, 45, 45, 109, and 128 days after injection. Four of the five chickens injected with dried blood developed leukosis; two died 27 and 76 days after injection, two were killed 35 and 76 days after injection.

It is evident from this experiment that the dried blood, kept in a sealed tube for 87 days in the ice box, contained much of the active agent, whereas fresh plasma of the same bird, passed through a Berkefeld filter, contained little.

Experiment 2.—Four chickens were inoculated with dried whole blood, taken from a chicken with leukemic lymphomatosis (No. 2643), and kept for 103 days in the ice box. From 12 to 83 mg. of dried blood taken up in Locke solution was injected into each bird (1 mg. of dried blood was equal to 0.01 cc. of fresh whole blood). All chickens appeared healthy when killed 157 days after injection. The fresh blood of the same chicken, injected in amounts of 0.1 cc. into chickens of the same size, caused leukosis in all four chickens injected.

Thus in this experiment drying of blood resulted in inactivation of the transmitting agent. Quantitative relations were ignored in both experiments, but are being considered in the experiments now under way.

Miscellaneous Experiments with Material Free from Viable Cells

Glycerination.—In one experiment five chickens were injected with from 0.07 to 0.7 cc. of whole blood, taken from a chicken with atypical leukosis (No. 2768), and kept for 65 days in approximately 80 per cent glycerin. Three of these chickens died of intercurrent diseases, 34, 44, and 86 days after injection, without showing any lesions suggestive of leukosis and two remained healthy and were killed 158 days after injection. Fresh blood from the same chicken caused leukosis in 10 of 12 chickens injected with from 1 to 4 cc.

In another experiment, loosely packed cells of a chicken with atypical leukosis (No. 2976) were kept for 113 days in glycerin (3 cc. cells plus 10 cc. concentrated glycerin c.p.) and were injected into five chickens in amounts corresponding to 0.5 cc. of cells. All of these birds remained healthy. All of five chickens injected with 0.25 to 0.5 cc. of fresh blood from the same chicken died with leukosis 43, 74, 82, and 83 days after injection.

Thus, attempts to preserve the transmitting agent in approximately 80 per cent glycerin were unsuccessful in two experiments.

Freezing.—Freezing at approximately −70°C. for 1/2 hour caused little if any deterioration of the transmitting agent. Three chickens were injected with 0.005 cc. of loosely packed fresh blood cells and three chickens with the same amount of cells after they had been kept at −70°C. for 1/2 hour. Two chickens of each group developed leukosis, those injected with fresh blood after 15 to 25 days, those injected with frozen blood after 29 days.

Technique.—0.5 cc. of the cells was sealed in a test-tube which was immersed in ether and kept for 1/2 hour in a thermos bottle containing one-half of its volume of solid carbon dioxide.

Experiments with mouse leukemia indicate that the death point of leukemic lymphocytes is, at temperatures below the freezing point, between −10° and −20°C. There is little evidence in favor of the view of Cramer and Foulds (21) that mammalian tumors are caused by invisible agents that share with the filterable agents of avian tumors the property of resisting freezing and thawing but do not share filterability and resistance to drying. Our experiments show that freezing at temperatures below −30°C. injures little if at all the filterable agents of leukosis of chickens, but destroys blood cells and destroys the ability of malignant lymphocytes of mice to transmit lymphomatosis (20). It is a matter of dispute whether there is a difference in resistance to low temperature between cells of vertebrates on the one hand and microorganisms and viruses on the other hand (*cf.* Rivers (22)); there are not enough experimental data to settle this question, for most, if not all, of the experiments on resistance of organisms to freezing were concerned with the process of freezing and thawing and did not record the actual temperature to which all cells were exposed. Our experiments suggest that freezing and thawing at temperatures below −30°C. for 30 minutes is a very good procedure to destroy living cells without inactivation of agents that transmit leukosis of chickens. It would be highly desirable to search with this procedure for the presence of agents in chicken tumors not transmissible by filtrates, for demonstration of filterability often fails because of technical difficulties such as association of the virus with larger particles retained by the filter.

Control Material

For comparison with the types of diseases caused by and characteristic of Strain 2, the incidence of tumors, leukosis, and related conditions occurring among all of the chickens kept in our animal rooms throughout these transmission experiments was analyzed (Table VII). This table includes 298 chickens that were inoculated with transmissible leukosis, Strain 1, 20 chickens used for miscellaneous transmission experiments, 14 chickens injected with various chemicals, and 16 uninjected chickens, all kept in our animal rooms during this period. No immature lymphocytes characteristic of Strain 2 have been seen in the blood smears of these chickens.

TABLE VII

Survey of All Chickens Kept in Our Animal Rooms during the Period of Experimentation with Strain 2

Chicken	No. of chickens in group	Free from leukosis and tumors	Type of leukosis				Sarcoma	Uncertain for leukosis	No. died of intercurrent disease	
			Erythroleukosis and myeloblastomatosis	Myelocytomatosis	Lymphomatosis	Atypical			Within 1 mo.	From 1 to 2 mos.
Uninjected	16	11	0	1	0	0	1	1	1	1
Injected with various chemicals	14	12	0	0	0	0	2	0	0	0
Transmission experiments										
Strain 1	298	120	137	0	0	0	1	1	23	16
Strain 2	431	209	10	17	72	38*	5†	8	38	36
Miscellaneous	20	16	0	0	1	0	0	0	1	1

* In 13 instances the type of leukosis was not determined.

† 2 instances of sarcoma occurred in association with leukosis.

One chicken, injected with tumor cells deriving from a chicken with spontaneous aleukemic lymphomatosis (given in the group of miscellaneous transmissions), died of aleukemic lymphomatosis. Attempted subpassages made from this bird into six chickens (given also among miscellaneous transmissions) were unsuccessful.

In none of the chickens injected with Strain 1 was myelocytomatosis found, but it was found in one of the uninjected controls. This spontaneous instance of myelocytomatosis was associated with endothelioma, the only presumably spontaneous tumor of this type I have ever seen. It is noteworthy that during the period of these investigations

(April to December, 1932) the birds were kept side by side in meshed wire cages, and in several instances birds injected with different material were kept in the same cage. Yet there is no evidence that Strain 1 became contaminated by Strain 2, or Strain 2 by Strain 1. Since December, 1932, the cages holding the birds injected with different strains of leukosis have been kept in separate groups but in the same room. Uninjected chickens have been kept as controls, some separate from and some among the inoculated chickens. No transmissible leukosis has so far (May, 1933) been observed among approximately 30 uninjected control chickens thus treated.

DISCUSSION

Are all types of leukosis observed among the passages of Strain 2 caused by a single agent?

The data presented indicate that lymphomatosis, myelocytomatosis, and endothelioma of fowls are transmissible diseases. Of 294 chickens injected with cell-containing material (Table IV), 131 developed leukosis, 60 of the latter had lymphomatosis, 14 myelocytomatosis, and 10 instances of leukosis were associated with endothelioma. Among 73 chickens inoculated with material free from viable cells (Table VI) 23 instances of leukosis were observed; 11 chickens developed lymphomatosis, 3 myelocytomatosis, and 1 instance of leukosis was associated with endothelioma. Since myelocytoma and endothelioma are rare as spontaneous diseases, it is probable that they too are caused by a filterable agent.

Indeed the experiments suggest that lymphomatosis, myelocytomatosis, and endothelioma are caused by the same agent. Whether transmissions are made from apparently pure instances of lymphomatosis or of myelocytomatosis, there occur instances of both lymphomatosis and myelocytomatosis among the successfully inoculated birds. Lymphomatosis usually occurs more often than myelocytomatosis. Endothelioma free from lymphomatosis and myelocytomatosis was not observed. Of 13 instances of endothelioma with giant cell formation, nine occurred among passages made from a leukotic fowl apparently free from this disease. Our examination of small pieces of tissue does not exclude the possibility that minute lesions of endothelioma escaped discovery at the postmortem examination. But one would expect a higher incidence of endothelioma from transfers made from apparently pure cases of that disease. Similarly, were different viruses responsible for lymphomatosis, on the one hand, and myelomatosis, on the other, one would expect among the successfully inoculated chickens a great incidence of the type from which the transfer is made, but this is not the case. Nevertheless, the evidence presented here does not exclude the possibility that we are dealing with an intimate mixture of viruses, and, for this reason, further efforts toward separation are being made. Transmission experiments are being conducted with washed cells of tumors composed of one type of cell, and cross-immunity tests, including tests with Rous sarcoma, are being made. The experiments thus far performed indicate that sarcoma can be grafted upon chickens that are resistant to repeated inoculations of leukosis Strains 1 and 2.

Another inference drawn from these observations is that transmission is accomplished mainly by the filterable agent and not by multiplication of the introduced cells.

Intramuscular or subcutaneous inoculations with this strain are, with rare exceptions, not followed by tumor formation at the site of injection, whereas tumors and leukemia of mammals (20) and Rous sarcoma of chickens, including endothelioma of Begg, produce tumors at the site of injection. After subcutaneous injection of tumor tissue derived from two spontaneous instances of Rous sarcoma (one occurred in an uninjected control chicken, one in a chicken inoculated with leukosis Strain 2) tumors developed readily at the site of injection. The agents of Rous sarcoma are capable of producing tumors in the subcutaneous tissue because the cells that they stimulate are present there, whereas the agents of leukosis have to reach blood-forming organs to find cells that can be stimulated by them. Lack of tumor formation after subcutaneous and intramuscular injection of leukemic lymphocytes or myelocytes supports the opinion that transmission is accomplished mainly by the filterable agent.

Morphologically, there is a similarity between endothelioma of Begg and endothelioma occurring among the passages of Strain 2. The former is not associated with leukosis and stimulates endothelium of young capillaries, at the site of injection, to neoplastic growth. Strain

2 does not stimulate endothelium at the site of injection, and endothelioma caused by it is always associated with leukosis.

The data presented by us show that there are at least two different agents that cause leukosis. Since the visceral alterations caused by the virus of neurolymphomatosis of Pappenheimer, Dunn, and Cone are leukotic in nature, we may regard their virus as a third virus that stimulates cells of the hemopoietic system to apparently unrestricted multiplication. The agent of Jármai that has affinity for the erythropoietic tissue only may represent a fourth type of transmissible leukosis.

The relation of neurolymphomatosis to this strain of lymphomatosis, which is also capable of infiltrating nerves and causing paresis or paralysis, will be discussed more fully in the report that follows. Suffice it to say here that the agent of neurolymphomatosis does not produce blood changes, whereas immature lymphocytes appear in the blood of most of the chickens successfully inoculated with our strain. Myelomatosis and endothelioma are not caused by the strain of Pappenheimer and coworkers, and lymphomatosis of viscera occurs only in 10 per cent of their paralyzed chickens. On the contrary, lymphomatosis of the viscera is the primary change produced by our Strain 2, and it is doubtful whether the two instances of paralysis without infiltration of viscera, occurring among the passages of our strain, are caused by the agent of Strain 2, for attempted transmission of one of these cases was unsuccessful. Infectious paralysis of Marek (*cf.* 16) and Seifried (16) is a primary inflammatory degenerative disturbance of the nervous system, and is unassociated with lymphomatosis of the viscera. Strain 2 is a primary neoplastic disturbance of hemopoeitic tissues with secondary invasion of the nerves, and neurolymphomatosis of Pappenheimer and coworkers is sometimes lymphomatosis of both viscera and nerves, and sometimes paralysis caused by inflammation of nerves unassociated with lymphomatosis of viscera.

Claude and Murphy (23) have compared the filterable agents of avian tumors with the substance described by Griffith and by Alloway that transforms one type of pneumococcus into another. Applying this analogy to Strain 2 one would expect that the filterable agent derived from myelocytomatosis would produce myelocytomatosis only, and that derived from endothelioma would produce endothelioma only, but the evidence presented shows that the type of leukosis produced is independent of that from which the agent is obtained.

The filterable agents of avian tumors and leukosis are growth stimuli; the disturbance caused by them depends on the type of cell attacked, on the character of the stimulus (agent), and on some additional unknown factors. Strain 2 is an example of a single agent that produces several types of diseases; whatever the type of the disease, the identity of the agent remains unchanged.

SUMMARY

A new transmissible strain of leukosis of chickens is described that causes (*a*) lymphomatosis with or without tumor formation, and with or without leukemia, (*b*) myelocytomatosis with or without leukemia, and (*c*) endothelioma. All these diseases are transmissible by material free from viable cells, and the available evidence indicates that they are caused by a single filterable agent.

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EXPLANATION OF PLATES

The blood smears were stained with a combination of Wright and Giemsa's solutions, the sections with hematoxylin-eosin. The magnifications given are approximate.

Plate 14

Fig. 1. Photograph of the ovary and kidneys of Chicken 2255 (spontaneous leukemic lymphomatosis). There are numerous tumor nodules in the kidneys; the ovary seems normal.

Fig. 2. Photograph of the liver of the same chicken. The liver is thickly studded with partly confluent tumor nodes.

Fig. 3. Blood smear of Chicken 2255 showing large numbers of basophile lymphocytes. × 450.

Fig. 4. Same with higher magnification (× 1500).

Fig. 5. Lymphocytes characteristic for Strain 2 in the blood of a chicken with subleukemic lymphomatosis. They are basophilic and contain small vacuoles and azure granules. × 1300.

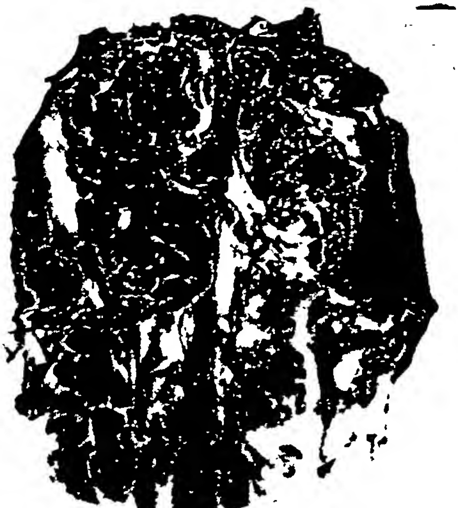
Plate 15

Fig. 6. The liver of Chicken 2255 showing extensive lymphomatosis infiltrations. × 90.

Fig. 7. The spleen of Chicken 2255 showing the character of infiltrations. × 270.

Fig. 8. Syncytial mass of tumor cells infiltrating the liver. × 700.

Fig. 9. The kidney of Chicken 2255 showing extensive lymphomatosis infiltrations. × 90.



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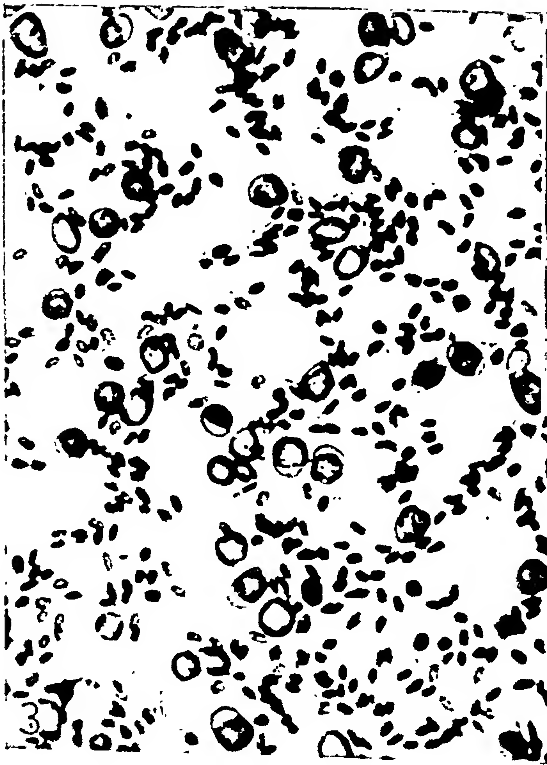

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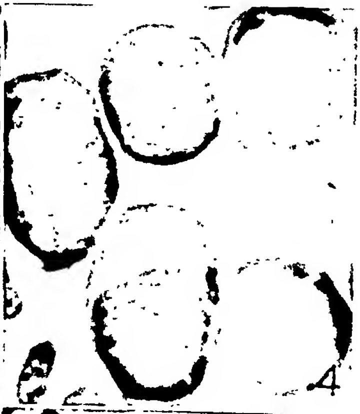

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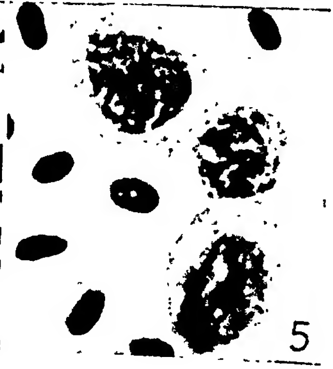

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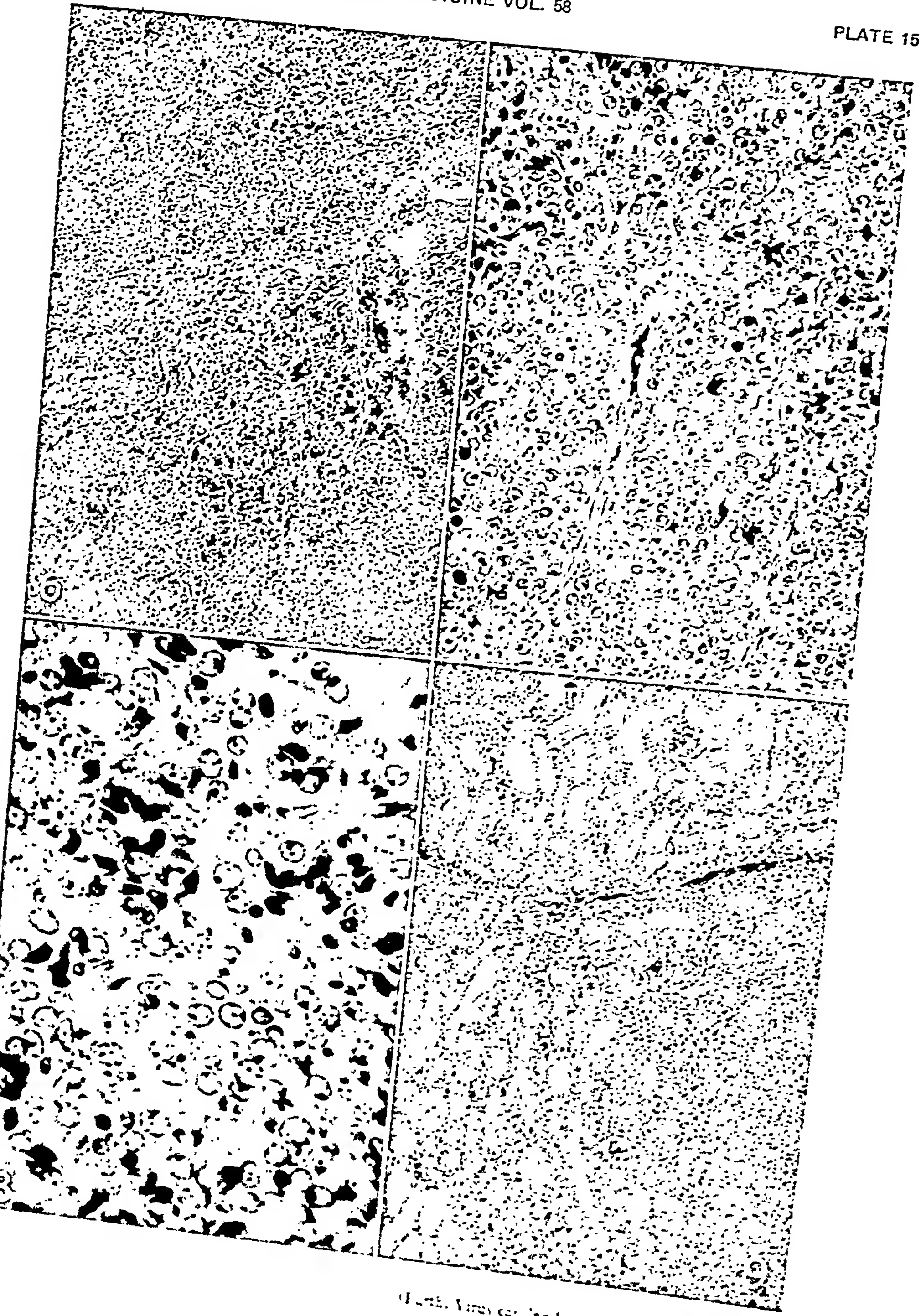
(Furth: Virus causing lymphomatosis of chickens. I)



(Furth: Virus causing lymphomatosis of chickens

VARYING HEMOLYTIC AND CONSTANT COMBINING CAPACITY OF STREPTOLYSINS; INFLUENCE ON TESTING FOR ANTISTREPTOLYSINS

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While investigating the antistreptolysin content of patients' sera according to Todd's method (1–3), we found that the hemolytic titer of one given streptolysin might vary considerably within a short period. The purposes of this communication are: first, to show that although the hemolytic titer of such a streptolysin does vary, its power of combining with antistreptolysin is constant under certain conditions; and second, to discuss the effect of this phenomenon upon the standardization of streptolysins which are to be used in testing sera for antistreptolysins.

Neill and Mallory (4) demonstrated that streptolysin may exist in at least two forms: (*a*) reduced and hemolytic, and (*b*) oxidized and non-hemolytic. They further showed that these two forms are easily reversible, one to the other, by oxidation and reduction. Such a reversibility would readily explain variations in hemolytic titer because the degree of hemolysis depends upon the amount of the reduced (hemolytic) form alone; such a reversibility, nevertheless, might not change the total streptolysin present in a given lot of hemolytic broth. It seemed to us that this hypothesis predicates a constant combining capacity of streptolysin with antistreptolysin even though the hemolytic strength might vary. In order to test it the following experiments were performed.

Large amounts of streptolysins Nos. 32, 33, and 35 were made. Each batch was divided into several different samples and each sample was subjected to different conditions, in order to obtain different hemolytic titers. In these experiments, the amount of hemolytic broth required to hemolyze completely 0.5 cc. of 5 per cent red blood cells in 1 hour was determined; this is known as 1 M.H.D. (minimal hemolytic dose) (1). Simultaneously, the amount of serum required to neutralize 0.5 cc. of hemolytic broth was determined. The results are shown in Table I.

In Table I it is seen that the amount of a given hemolytic broth required to hemolyze completely 0.5 cc. of 5 per cent red blood cells varied within wide limits, while, on the other hand, 0.5 cc. of one lot of streptolysin was always neutralized by a constant amount of one serum. Thus, in the case of streptolysin No. 32, 0.04 cc. of Sample B completely hemolyzed 0.5 cc. of 5 per cent red blood cells, though 0.40 cc. of Sample C was insufficient to accomplish this. Nevertheless, 0.5 cc. of both Samples B and C required 1/120 cc. of serum for neutralization; hence, the hypothesis above outlined was substantiated.

TABLE I

Constant Combining Capacity Compared with Varying Hemolytic Strength of Three Different Lots of Streptolysin

Strepto-lysin No.	Conditions to which streptolysin was subjected	Amount of hemolytic broth required to hemolyze 0.5 cc. of 5 per cent red blood cells	No. of M.H.D. per 0.5 cc. of hemolytic broth	Amount of serum required to neutralize 0.5 cc. of hemolytic broth
		cc.		*cc.*
32	A. Freshly opened, prepared, and reduced 2 wks. previously	0.18	2.8	1/120 serum Eis
32	B. A exposed to room temperature and air 8 hrs.	0.04	12.5	1/120 " "
32	C. Freshly opened, reduced 2 days previously	>0.40	<1.2	1/120 " "
32	D. 1 part A, 3 parts unreduced	0.18	2.8	1/120 " "
32	E. Reduced day of using, exposed to air and room temperature 8 hrs.	0.06	8.3	1/120 " "
33	A. Freshly opened, prepared, and reduced 1 wk. previously	0.20	2.5	1/250 " "
33	B. A after 8 hrs. unsealed at room temperature and 16 hrs. unsealed in the ice box	0.08	6.2	1/250 " "
35	A. Immediately after being made and reduced	0.14	3.6	1/100 " Val
35	B. A after 24 hrs. in the ice box	0.08	6.2	1/100 " "

The constancy of combining power compared with the variability of hemolytic power made it desirable to determine whether a streptolysin, completely non-hemolytic because in the unreduced state, still had the same combining capacity as when it was reduced. By setting up an experiment in stages, it was demonstrated that unreduced (non-hemolytic) streptolysin No. 32 had practically the same combining capacity as is shown in Table I.

Repeated experiences of this type made it obvious that one must find a method for standardizing streptolysin other than that originally recommended by Todd (1), and which he later found it necessary to modify. Theoretically 1 streptolysin unit would be neutralized by 1 antistreptolysin unit. Todd (1, 2) defined 1 antistreptolysin unit in a given serum as the amount that would neutralize 2½ minimal hemolytic doses of streptolysin when 1 minimal hemolytic dose (M.H.D.) would completely hemolyze 0.5 cc. of 5 per cent rabbit red blood cells. According to this definition 1 combining unit of streptolysin should correspond to 2½ hemolytic doses. That such a correspondence does not constantly exist is shown in Table I and is even better illustrated in the following experiment.

TABLE II

Influence of Both Varying and Keeping Constant the Dose of Hemolytic Broth

Streptolysin No. 32	Condition of streptolysin	Amount of hemolytic broth producing complete hemolysis	Amount of hemolytic broth used in test	No. of M.H.D.	Amount of serum required for neutralization
		cc.	cc.		cc.
A. Using 2½ M.H.D. as a constant	Immediately after reduction	0.2	0.5	2½	1/250
	1 hr. after reduction	0.1	0.25	2½	1/444
	24 hrs. after reduction, kept cold	0.04	0.1	2½	1/1,200
B. Using constant amounts of hemolytic broth regardless of hemolytic titer	Immediately after reduction	0.2	0.5	2½	1/250
	1 hr. after reduction	0.1	0.5	5	1/250
	24 hrs. after reduction, kept cold	0.04	0.5	12½	1/250

Streptolysin No. 32 was reduced *in vacuo* with 0.1 per cent sodium hydrosulfite. Portions of this streptolysin kept in the ice box during the period of the experiment were tested simultaneously for hemolytic and combining power at the following periods: immediately after reduction, 1 hour, and 24 hours later. The results are shown in Table II.

Table II illustrates the unreliability of standardizing the combining capacity of streptolysins by their hemolytic strength. It further shows the necessity of discarding the 2½ M.H.D. standard and adopting one based on the total streptolysin content of a given reagent. If attempts had been made to standardize this lot of streptolysin by the 2½ M.H.D. method, its combining capacity would have apparently varied almost 500 per cent. When, on the other hand, the amount of streptolysin-containing broth was kept constant in spite of a varying hemolytic titer, its combining capacity was found to be constant.

Todd (1) stated that it was impossible to titrate streptolysins accurately by their hemolytic capacity because of technical difficulties in determining their hemolytic strength, and also because non-hemolytic streptolysin had some power of combining with antistreptolysin. To test the latter point he used streptolysin so altered by long exposure to air that it could not be reduced to the hemolytic form. This altered streptolysin had lost all of its hemolytic power and well over half of its combining capacity. These changes were apparently irreversible (1, 5). In contrast to this, our experiments demonstrate still another reason for titrating streptolysins according to their combining capacity; namely, the easy and rapid reversibility of the non-hemolytic to the hemolytic form.

It will also be observed from Table II that reduction progressed over a period of 24 hours. Such an observation suggested that it might be possible to push reduction to a point of completion, where all of the unreduced (non-hemolytic) form had been converted into the reduced (hemolytic) form. In such a condition, there should be a rough parallelism, at least, between the hemolytic titer and combining capacity of all streptolysins thus treated. We have, however, been unable to obtain any such parallelism with regularity, possibly because of the presence of varying amounts of streptolysin so altered that it could no longer be reduced to the hemolytic form, although it still retained some power of combining with antibody.

Combining Stability of Streptolysin

All of the data so far presented, suggested that it might be possible to prepare and reduce a large amount of streptolysin at one time, standardize it with a number of sera of known antistreptolysin strength, and use it as a standard reagent over a considerable period. The stability of such a reagent is illustrated in Table III.

TABLE III

Comparative Constancy of Combining Power of Various Lots of Streptolysin

Streptolysin No.	Date of preparation and reduction	Date of test	Amount of serum required to neutralize 0.5 cc. of streptolysin	Serum
			cc.	
26	Aug. 26, 1932	Oct. 28, 1932	1/160	Wei
26	" 26, 1932	May 13, 1933	1/133	"
26	" 26, 1932	Oct. 20, 1932	1/111	Sch
26	" 26, 1932	May 13, 1933	1/100	"
26	" 26, 1932	Oct. 28, 1932	1/40	McE
26	" 26, 1932	May 13, 1933	1/50	"
33	Jan. 25, 1933	Feb. 5, 1933	1/62	Br
33	" 25, 1933	May 14, 1933	1/62	"
33	" 25, 1933	Jan. 26, 1933	1/300	Eis
33	" 25, 1933	Feb. 1, 1933	1/260–1/300	"
33	" 25, 1933	Feb. 5, 1933	1/260	"
33	" 25, 1933	May 14, 1933	1/300	"
33	" 25, 1933	Feb. 1, 1933	1/250	Val
33	" 25, 1933	Feb. 3, 1933	1/240	"
33	" 25, 1933	Feb. 5, 1933	1/225	"
33	" 25, 1933	Feb. 6, 1933	1/225	"
33	" 25, 1933	May 14, 1933	1/240	"

The variations noted in Table III are within the limits of error of the method employed.

In order to maintain this stability it has been necessary to keep the flasks containing the reduced streptolysin in the refrigerator and well sealed with vaseline. Certain lots not sealed, even though kept in the cold, have become weaker in both hemolytic and combining strength; and while this has not been a constant phenomenon, it has occurred often enough to make advisable the use of freshly opened streptolysin.

To determine further the effect of exposure to air a number of flasks of the reagent were opened, part of the contents removed, and the flasks immediately sealed and replaced in the ice box. Tests made at the time of original opening and later are presented in Table IV.

The results in Table IV indicate that reduced streptolysin opened and resealed may be safely used within a few days, but that less reliance may be placed on such reagents kept longer periods.

TABLE IV

Constancy of Combining Power of Streptolysin Opened and Resealed

Streptolysin No.	Serum	Amount of serum required to neutralize 0.5 cc. of streptolysin at time of original opening and resealing	Interval between tests	Amount of serum required at time of second testing
		cc.		*cc.*
33	Wer	1/180	3 mos.	1/200
34	Ste	1/33	1 mo.	1/30
34	"	1/33	2 days	1/33
34	N-99	1/100	1 mo.	1/100
34	Wel	1/140	1 day	1/125
34	"	1/140	2 days	1/140
34	Rap	1/140	2 "	1/140
35	Wel	1/166	3 mos.	1/200

DISCUSSION

The data here presented indicate that for the purpose of determining the antistreptolysin content of a given serum it is essential to know the combining capacity of the streptolysin used rather than to determine its exact hemolytic power in terms of minimal hemolytic dosage.

The technical difficulties in the actual test arise from the fact that streptolysin plays at least two different rôles *in vitro*: first, that of a substance which combines with antibody, apparently in multiple proportions, in the first stage of the reaction, and second, that of an indicator which makes itself manifest by lyzing erythrocytes in the second stage. The streptolysin exerts the first function either in the oxidized or reduced state, and the second function only in the reduced form. To titrate the exact point where streptolysin and antistreptolysin have completely combined one with the other, it is necessary to have additional tubes *containing some uncombined, reduced (hemolytic)* streptolysin which will lyze the erythrocytes at all points in excess of that where the total streptolysin has been rendered completely inert by the antibody. Any excess amount of free active lysin will indicate where this point lies; hence the last tube where no hemolysis occurs shows where the union of antigen (streptolysin) and antibody has been complete. The important factor is that this streptolysin must be free in order to attack the erythrocytes and active in order to lyze them.

Originally Todd (1, 2) produced streptolysin by means of growing the streptococci anaerobically in a yeast extract reinforced broth sterilized by filtering through a Chamberland candle. The reagent thus produced seemed to contain about 2½ M.H.D. per 0.5 cc. of broth and to possess equivalent hemolytic and combining powers. The same strain of streptococcus, however, when grown aerobically in broth reinforced with dextrose, certain buffers, and salts (6, 7) has yielded much stronger concentrations of streptolysin per cubic centimeter of medium. Reagents produced in the latter way have not shown any constant parallelism between hemolytic and combining powers. The influence of varying degrees of oxidation and reduction in this media, with corresponding variations in amounts of hemolytic, compared with non-hemolytic streptolysin, has made it necessary to change the methods of standardization of the reagent. If precise and constantly reproducible conditions for complete reduction of streptolysin to an actively hemolytic form could be determined, the standardization of streptolysin in terms of 2½ M.H.D. would be possible. Up to the present time, however, we have been unable accurately to define these conditions.

The fact, on the other hand, that a given amount of streptolysin kept under certain conditions has a constant combining capacity which is doubtless fixed regardless of whether it is in the reduced or oxidized form, or in a combination of the two, makes the standardization of a given lot of streptolysin more simple. The additional fact that the reagent maintains its combining capacity at a fairly constant level, provided it is kept sealed and cold, makes easier the preservation of a quantity of standardized reagent.

Todd (8) has suggested that a serum containing a known number of antistreptolysin units be universally employed as a standard;[1] but up to the present no such standard serum has been available. In order to keep the same system of units already employed by Todd, we have standardized our streptolysin with a number of sera furnished by Dr. Coburn, as described under Technique 4; duplicates of these sera had been tested by Todd. About 10 per cent of them showed wide variation from the results obtained by Todd, who (8) has suggested to us that the discrepancies are due to contamination of the sera; for as a rule we have found that sera kept sterile and cold have maintained quite constant their antistreptolysin content.

After the completion of this work Todd's latest paper (5) appeared showing that both reduced and unreduced streptolysins are active antigens *in vivo*, in that they both induce the production of antistreptolysin when injected into animals; and incidentally he showed both here and in his original paper (1) that irreversibly oxidized streptolysin combined with antistreptolysin, though in different proportions than did reduced streptolysin. Furthermore, streptolysin produced in serum broth, while acting as an antigen *in vivo* would not combine with antistreptolysin *in vitro*, even though it would lyze erythrocytes. This work furnishes additional proof of the necessity of determining accurately the dosage and conditions under which a streptolysin will combine with antistreptolysin if it is to be used for measuring the amount of the antibody in a given serum.

SUMMARY

Certain properties of streptolysin in respect of hemolytic power and capacity for combining with antistreptolysins have been determined. The hemolytic strength may vary markedly, while under suitable conditions the combining power is constant.

This stability of combining capacity makes it possible to prepare a

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[1] By antistreptolysin units Todd means the reciprocal of the fraction of a cubic centimeter of serum that will neutralize a standard amount of streptolysin. Obviously this amount of streptolysin must have been previously standardized according to the method herein described.

large amount of streptolysin, standardize it against sera of known antistreptolysin strength, and use it as a reagent over a considerable period for testing the antistreptolysin content of unknown sera. A modified technique for making these tests is described.

APPENDIX

Technique

So many modifications of Todd's original technique (1) have been introduced that the following steps are described.

1. Making the Broth (6, 7).—Fresh beef heart is freed of fat, finely ground, mixed with tap water in the proportion of 1 pound per liter, infused 16 hours in the ice box, heated slowly to 85°C., where it is maintained for 30 minutes. It is filtered through Pratt-Dumas filter paper, the meaty pulp squeezed dry, and 20 gm. of Difco proteose peptone dissolved in each liter of infusion. The reaction is adjusted to pH 8; the broth is then filtered through No. 12 Whatman filter paper, and placed in flasks. It is sterilized in the Arnold apparatus on 3 successive days. Each liter is then reinforced with the following solution which is sterilized by filtering through a Berkefeld candle before addition to the meat infusion: dextrose 2 gm., $NaHCO_3$ 2 gm., NaCl 2 gm., $Na_2HPO_4 \cdot 12\ H_2O$ 1 gm., water 50 cc. The reinforced broth should be incubated to insure sterility.

2. Production of Streptolysin.—The broth, warmed to 37°C., is heavily inoculated with an actively growing culture of a suitable hemolytic streptococcus[2] incubated for 16 hours—then filtered through a Chamberland F candle, with the precaution of discarding the first 100 cc. of filtrate.

This filtrate is then reduced *in vacuo* by adding 1 gm. of freshly ground sodium hydrosulfite to each liter, and maintaining a high vacuum until only a few bubbles are given off. The addition of NaOH as originally advised by Todd has usually been found superfluous, as the pH of the streptolysin after reduction is about 7.4 to 7.8. The essential requirement is that the final reaction after complete reduction remain slightly alkaline. The streptolysin is placed in tubes or small flasks and sealed with a layer of warm (not hot) sterile vaseline, about 5 cm. thick, and stored in the ice box.

3. Titration of Hemolytic Power.—Beginning with 0.5 cc. of hemolytic broth, decreasing quantities in steps of about 10 per cent are placed in a series of Wassermann tubes. Physiological saline is added to make the total volume in each tube equal 1.5 cc. Then 0.5 cc. of 5 per cent washed red blood cells (rabbit) is added, quickly and thoroughly shaken, and incubated 1 hour. The last tube showing complete hemolysis contains 1 M.H.D.

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[2] We have employed Strain W P R L furnished by Dr. Todd.

4. *Determining the Standard Combining Power.*—In a series of tubes the streptolysin is diluted by 10 per cent decrements; the volume in each is made 0.5 cc. with physiological salt solution, then 1 cc. dilution of a known standard serum is added, the serum having been diluted so that 1 cc. equals 1 unit. The mixture is shaken immediately and after 15 minutes, is incubated 1 hour, when readings are made. The last tube showing no hemolysis represents the point of complete neutralization, or in other words, 1 combining unit. This testing should be done with several sera of known unit value, high, low, and medium, before the standard dose of a given streptolysin is fixed.

5. *Testing Human Sera for Antistreptolysin.*—Todd's technique (2) calls for a preliminary testing with 1:10, 1:100, and 1:1,000 dilutions of the serum and a second titration using 0.1 cc. increments of the dilution indicated by the preliminary test. Measured in units this gives a very uneven gradation. We have, therefore, adopted a system whereby the gradations consist of 50 units from 50 to 500, 100 units from 500 to 1,000, and 200 units from 1,000 to 2,000. As the majority of sera contain less than 500 units, this is the usual limit; and a second testing with the higher dilutions is only occasionally necessary. Where the approximate range is known still fewer tubes are required.

Protocol 1

Scheme for Diluting Serum to Obtain a Given Number of Units of Antistreptolysin per Tube

Normal salt solution	0.0	0.5							
Serum 1:25 dilution	1.0	0.5							
Tube represents antistreptolysin units	25	50							
Normal salt solution		0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Serum 1:100 dilution	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cc. employed in test	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Tube represents antistreptolysin units	100	150	200	250	300	350	400	450	500
Cc. employed in test					0.5	0.5	0.5	0.5	0.5
Tube represents antistreptolysin units					600	700	800	900	1,000
Cc. employed in test					0.25	0.25	0.25	0.25	0.25
Tube represents antistreptolysin units					1,200	1,400	1,600	1,800	2,000

Two dilutions of serum to be tested, 1:25 and 1:100 respectively, are made with physiological salt solution, and subsequently diluted as shown in Protocol 1.

Where necessary the volume of dilution in each tube is made up to 1 cc. with physiological salt solution. The previously standardized streptolysin is diluted with physiological salt solution so that 0.5 cc. represents 1 combining unit as previously determined in Step 4. This amount is added to each tube of diluted serum, well mixed and incubated in the water bath at 37°C. for 15 minutes, then 0.5 cc. of 5 per cent washed red blood cells (rabbit) is added, well shaken immediately and again after 15 minutes' incubation. The tubes are incubated for a total of 60 minutes, and the readings made immediately. The last tube showing no hemolysis represents the number of units of antistreptolysin in the serum tested. Control series containing no serum, serum of known low titer, and serum of known higher titer are introduced each day; thus a constant check on the combining capacity of the streptolysin is maintained; and when it varies appreciably from that originally determined the tests for the day are discarded.

Todd (8) has suggested to us that the streptolysin be diluted with broth similar to that in which it was originally made; but we have found that some lots of broth apparently inactivate the streptolysin. Physiological salt solution, on the other hand, has proven completely indifferent. It is advisable to dilute only approximately the amount of streptolysin necessary for 1 day's work, because of the danger of deterioration after dilution. A new lot of streptolysin may be conveniently standardized by running parallel tests with the new and with the standard streptolysin with various sera at the time the antistreptolysin contents of these sera are determined.

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STUDIES ON AN UNCOMPLICATED CORYZA OF THE DOMESTIC FOWL

I. THE ISOLATION OF A BACILLUS WHICH PRODUCES A NASAL DISCHARGE

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Under natural conditions the domestic fowl is subject to a number of infections which affect the nasal passages and result in a discharge from the nares. Laryngotracheitis, fowl pox, and fowl cholera may be accompanied by an inflammation of this character but in these cases the coryza is generally a transient manifestation which precedes the appearance of lesions elsewhere. The fowl is also susceptible to an uncomplicated coryza, not unlike the common cold of man, in which the inflammatory reaction is limited to the mucosa of the nasal passage and the communicating portions of the orbital tract. This form of coryza, which is here considered, is probably identical with the condition known as catarrhal roup in the older literature and later designated contagious catarrh.

De Blieck[1] recently studied a fowl coryza of this type in Holland and isolated a hemophilic bacillus from a blood agar plate streaked with nasal exudate from a naturally affected bird. He stated that the organism resembled the human influenza bacillus and named it *B. haemoglobinophilus coryza gallinarum*. Normal fowl injected intranasally with pure cultures of the bacillus regularly developed a coryza. McGaughey[2] in England reported the isolation of a para-influenza bacillus, dependent only on V factor for growth, from the upper respiratory tract of fowl affected with coryza and also from normal birds. The bacillus was not pathogenic for fowl upon subcutaneous or intravenous injection. Intranasal injection was evidently not carried out. The significance of the organism as the cause of the coryza was considered to be uncertain.

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[1] De Blieck, L., *Vet. J.*, 1932, 88, 9.

[2] McGaughey, C. A., *J. Comp. Path. and Therap.*, 1932, 45, 58.

Isolation and Cultivation

The present work on fowl coryza was begun in the fall of 1931.[3] The disease was established in a group of normal birds by the introduction of nasal exudate from naturally affected fowl obtained on a nearby poultry farm and was subsequently maintained by transferring exudate to additional birds from time to time.[4] The material was injected into the palatine cleft and its introduction was followed after a short incubation period by an inflammation of the nasal passages and a discharge from the nares.

Most of the experimental fowl were drawn from a flock of Rhode Island Reds maintained for many generations at The Rockefeller Institute and known to be free from respiratory disease. As the work advanced it became necessary to use Rhode Island Reds from another flock and also White Leghorns but in both cases the birds were from disease-free stocks. The infected birds were kept indoors in cages or enclosed runways which were maintained under a strict quarantine.

Exudate from the experimentally affected fowl was first studied by ordinary bacteriological methods. A 1.5 mm. loop of the material removed from either the nares or the nasal canals was streaked over the surface of an open plate of 10 per cent horse or chicken blood agar. This examination served only to catalogue the bacterial flora of the affected air passages. Exudate withdrawn after the 2nd day of the coryza generally contained large numbers of bacteria, a single loopful often giving a nearly confluent growth of small colonies. Gram-positive cocci, diphtheroids, and Gram-negative bacilli predominated but rarely with any approach towards the pure growth of a particular organism. The former group included staphylococci, a greenish haloed streptococcus, a tetrad, and unidentified micrococci. The latter group embraced some 10 unidentified species and in addition several strains of a para-influenza bacillus. Bacilli of the Pasteurella group were not encountered. Representative cultures of the Gram-negative bacilli were injected into the palatine cleft of normal fowl and failed in all cases to produce a coryza. Most of these bacteria

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[3] A preliminary report of the early work was presented in *Proc. Soc. Exp. Biol. and Med.*, 1932, 30, 306.

[4] The original coryza cases were obtained by Dr. O. Seifried and Dr. C. Cain.

may be isolated from the nasal mucosa of normal birds but are present in much smaller numbers.

Exudate diluted with bouillon and filtered through short Berkefeld V candles was likewise innocuous.

As a control on the sterility of the filtrates a 0.5 cc. portion was added to fluid horse blood at the base of slanted nutrient agar. There was no macroscopic evidence of growth in these tubes. Fluid removed from the base of such a tube, after it had been incubated at 37°C. for 4 days, was injected into the palatine cleft of a normal fowl. 2 days later the injection was followed by a typical discharge from the nares. This was the first instance in which a nasal discharge had been produced in the absence of exudate. The uncultured filtrate in this case was non-infective, as usual. Exudate was removed from the nasal passages of the affected bird, filtered through the same candle after sterilization, and portions before and after cultivation injected in normal birds. Identical results were obtained with this series and again with a third in which exudate from the second bird was utilized. A repetition of the entire experiment modified only by the use of fluid chicken blood in the culture medium yielded similar findings throughout.

In all cases the birds which had received filtered exudate before cultivation remained normal, whereas those which had received the filtrate after cultivation, in the presence of fluid blood, developed a coryza.

In the experiments described above exudate was removed from the nasal passages, at autopsy, and rubbed up with a little bouillon in a glass tissue grinder. The suspension was diluted to a volume of approximately 5 cc. with bouillon and rapidly filtered through a 3.5 cm. Berkefeld V candle. 0.5 cc. portions of the filtrates were used in the inoculation of culture tubes, which were generally incubated at 37°C. for 24 to 48 hours, and in the injection of fowl. Blood agar plates were streaked with exudate prior to filtration and in all cases showed many colonies of miscellaneous bacteria.

As noted above, there was no macroscopic evidence of bacterial growth in the incubated tubes of filtered exudate, the fluid from which was infective. At first, some difficulty was experienced in detecting bacteria microscopically. It was shortly found, however, that films made from the fluid portion of the medium, not later than the 2nd day and after the tube had been shaken to disperse the sedimented red blood cells, regularly showed small Gram-negative bacilli. The supernatant portion of the fluid blood remained clear for several days and prior to shaking contained only an occasional bacillus. Upon prolonged incubation the fluid becomes turbid due to the precipitation of serum constituents. After the 2nd day of incubation the bacterial cells tended to fragment and lose their staining properties. In hanging drop preparations made from 24 hour old cultures the bacilli showed no motility. On open blood agar plates streaked with fluid from both young and old cultures and examined daily under low magnification there was no evidence of colonization.

Cultivation of the specific bacillus was continued by serial transfer to horse blood agar slants at weekly intervals. Normal birds were injected with fluid from such cultures from time to time and in every case through the 25th subculture the injection was followed by a coryza. A total of 35 birds varying from 5 weeks to 2 years in age but mostly between 8 and 12 weeks of age, were employed in these experiments which were carried out between January and July.

No differences were noted in the inflammatory changes produced by exudate and by cultures of the specific bacillus. There was a difference, however, in the course of the 2 coryzas, the nasal discharge continuing for a longer time in birds injected with exudate. The average duration of symptoms was approximately 11 days in 20 cases of the "exudate" coryza and 5 days in 20 cases of the "bacillary" disease.

Weekly transfers of the organism were made during the summer of 1931 but the maintenance of the coryza in fowl was discontinued. In September the infectivity of the 35th subculture was tested by intranasal injection, with the finding that the organism had become avirulent.

Before setting out to recover a new strain of the bacillus, an attempt was made to perfect the method of isolation. Differential filtration, while a useful procedure, is not suitable for routine examinations. Only certain candles function in that capacity and an adequate supply cannot be kept on hand.

It was found that the avirulent organism would colonize on the surface of blood agar plates that were tightly sealed. For this purpose a closely fitting cover of modeling clay was employed instead of the usual glass lid. Small, slightly rounded, clear colonies were produced on plates sealed in this way. With the aid of a dissecting microscope, transfers could be readily made from individual colonies to blood agar slants.

The covers used in sealing the plates were made from one of the commercial modeling clays. A ball of clay was pressed into a circular disk about 5 mm. thick and 1 cm. larger than the bottom half of a Petri plate. The projecting portion was moulded to form a raised rim or flange. A sterile paper disk was used to cover the inner portion of the clay cover, the inoculated dish inverted over it, and the flange firmly pressed to the side of the dish. The medium is thus in contact only with sterile surfaces and accidental contamination rarely occurs. The cover is readily removed intact by running a spatula inside the flange and may be used over and over again.

This method of isolation was employed with birds which had been injected with exudate from naturally infected fowl. The latter were obtained in October, 1932, on the same poultry farm from which the original cases had been secured during the preceding fall. Exudate was removed from the nasal canals of the experimentally infected fowl on the 1st or 2nd day of the coryza and streaked on plates, from which the specific bacillus was readily isolated. A coryza was regularly produced in normal birds by the intranasal injection of the organism in pure culture. The bacilli were again obtained on sealed plates prepared from the exudate of these fowl.

The incubation period of both the "exudate" and "bacillary" coryzas was 24 to 48 hours as in the case of those of the preceding year. The duration of the second "exudate" coryza was much longer however, than that of the earlier corresponding coryza. Symptoms regularly continued for 2 months or longer. The difference between the "exudate" and "bacillary" coryzas with respect to the duration of symptoms was more marked than in the case of the respective types of the preceding year. The average period in 15 cases of the second "bacillary" coryza was 11 days. It may be noted that White Leghorn fowl were used in addition to Rhode Island Reds in the above experiments. No difference was observed in the susceptibility of the 2 varieties.

Aside from variations in the duration of the disease, no differences were noted in the symptoms or the pathology of either the "exudate" or the "bacillary" forms of the coryzas studied in 1931 and 1932, respectively.

The experimentally infected birds showed a unilateral or a bilateral nasal discharge after a short incubation period. An initial unilateral discharge generally became bilateral as the coryza advanced. Early in the disease the exudate was largely a thin mucus which was relatively poor in cells. Later the mucus became thick and tenacious and mixed with large numbers of leucocytes and epithelial cells. Small cheesy granules made up largely of bacteria were also present in some of the advanced cases. The exudate was rarely limpid enough actually to drop from the nares but tended, rather, to collect there forming a plug which partially occluded the lumen. After the first few days the exudate was generally covered by a crust of dust and grain particles. At autopsy, a large volume of exudate was commonly found in the nasal passages and not infrequently in the orbital sinuses. Aside from the increased secretion of mucus the nasal mucosa showed little or no gross evidence of injury.

There were no other consistent manifestations or symptoms in the infected fowl. Occasionally the inflammation extended to the external periorbital tissues, with a transient watery discharge. At autopsy, a few birds showed a catarrhal inflammation of the trachea. There was no specific mortality and in most cases the infected birds did not appear ill. The majority of the birds showed no respiratory abnormalities; a few birds with a heavy bilateral discharge breathed through the mouth with the beak open and in those with an involvement of the trachea respiration was accompanied by a distinct gurgling sound. In the case of the persistent "exudate" coryza, growth and egg laying were noticeably retarded.

The appearance of naturally affected fowl was often quite different. Some of the birds received from the poultry farm where the coryza was endemic showed emaciation, diarrhea, a labored audible respiration, and edema or inflammation of the periorbital tissues. The latter was characterized by either a copious purulent discharge or a massive accumulation of exudate in the orbital cavity. In our experience the transference of nasal exudate from such birds to normal fowl maintained under controlled conditions was followed only by the development of a coryza.

DISCUSSION

The passage of the specific organism through certain coarse Berkefeld candles, which held back other bacteria, was a particularly fortunate circumstance in view of its inability to colonize on open blood agar plates. This peculiarity in growth obviously precluded plating, under ordinary conditions, as a method of separating the bacillus from the miscellaneous bacteria present in the nasal exudate of fowl affected with coryza.

The filtered exudate after cultivation in a suitable fluid medium showed intact bacterial cells in an uncontaminated condition. The precursors of these cells, present in the filtrate before cultivation, were incapable of producing a coryza in normal fowl, either by reason of insufficient numbers or of an altered biological state. After cultivation the filtered exudate was regularly infective.

Earlier attempts to induce colonization of the bacillus on solid media had been unsuccessful and the evidence that only one organism was present in filtrates from different birds was somewhat circumstantial. The subsequent discovery that colonization could be initiated by sealing the plates used for cultivation furnished a useful method of isolation and by the uniformity of the colonies supplied additional evidence that the same organism was present throughout.

The question which arises concerning the relationship of this bacillus to the one isolated by de Blieck[1] cannot be answered at present. The latter organism was described as resembling *B. influenzae* of man but a detailed statement of its growth requirements was not made. It apparently colonized on open blood agar plates. In the morphology of its cells and colonies the present bacterium bears a resemblance to the influenza bacillus and like it is soluble in bile. Unlike the latter, however, it fails to grow in bouillon containing X and V factors of plant origin, suggesting that its growth requirements may not include these accessory factors; and it fails to colonize on open blood agar plates. The present organism is readily differentiated from the para-influenza bacillus which was occasionally isolated from the nasal exudate of fowl affected with coryza. The latter bacillus required V factor only in the medium of growth, as did the strains isolated by McGaughey,[2] and it grew well in bouillon enriched with a plant extract instead of blood. There appears to be no good reason for regarding the present bacterium as an influenza bacillus, although it probably belongs to the group of hemophilic bacteria which includes that bacillus. Its final classification as well as a detailed description of its characters is, however, held in reserve.

SUMMARY

By a method combining filtration and cultivation an unidentified Gram-negative bacillus was isolated from the nasal exudate of fowl experimentally infected with an uncomplicated coryza. Isolation was accomplished by cultivation on sealed blood agar plates after unsuccessful attempts to produce colonies on open plates. Injection of the organism into the palatine cleft of normal birds was regularly followed by an inflammation of the nasal mucosa and a discharge from the nares. A para-influenza bacillus which was also recovered from the nasal tract of affected fowl was innocuous. Certain cultural characters of the bacillus, bearing on its classification, are discussed.

STUDIES ON AN UNCOMPLICATED CORYZA OF THE DOMESTIC FOWL

II. THE RELATION OF THE "BACILLARY" CORYZA TO THAT PRODUCED BY EXUDATE

BY JOHN B. NELSON, PH.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, June 13, 1933)

The isolation of a Gram-negative bacillus from the nasal exudate of fowl experimentally infected with an uncomplicated coryza is reported in the preceding paper.[1] The introduction of this organism into the palatine cleft of normal fowl was regularly followed by an inflammation of the upper air passages with a discharge from the nares. It was of importance to determine whether any infectious agent other than the specific bacillus is etiologically associated with the disease. The observation that the coryza produced by injection of the bacillus regularly ran a shorter course than that produced by exudate had suggested that a second agent might be involved. The results of protection tests carried out to test this possibility are here reported.

EXPERIMENTAL

Seven birds that had recovered from the coryza produced by the 1931 strain of the specific bacillus were subsequently reinjected with cultures of the same strain. It may be noted that the coryza studied in 1931 (Coryza I) was characterized by a relatively short course which averaged 5 days in the case of the "bacillary" form and 11 days in that produced by exudate. The interval between the disappearance of symptoms and the second injection of the organism varied from bird to bird, with extremes of 14 and 26 days. The reinfected fowl were examined daily during a period of 2 weeks and 6 of them remained normal throughout that interval. At autopsy, there was no evidence of an inflammation of the nasal mucosa. After an incubation period of 48 hours the seventh bird developed a coryza which lasted for 7 days. In this case the interval which preceded the second injection was 14 days.

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[1] Nelson, J. B., *J. Exp. Med.*, 1933, 58, 289.

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It was apparent from the above experiment that recovery from the "bacillary" coryza was attended by a considerable degree of immunity to reinfection with the bacillus. Reciprocal protection tests were then carried out to determine whether birds which had recovered from the "bacillary" disease were immune to reinfection with exudate and *vice versa*.

Two groups of 4 birds, confined in individual cages in separate quarantine units, were employed in this experiment. 0.5 cc. amounts of 24 hours old horse blood agar cultures of the specific bacillus were injected into the palatine cleft of the birds in one group. 0.5 cc. amounts of exudate, withdrawn from several fowl previously infected with "exudate" coryza, were similarly injected in the birds of the second group. After recovery from the initial coryza the birds of the first group were injected with exudate and those of the second with cultures of the bacillus. The interval between the disappearance of symptoms and the second injection varied from bird to bird with extremes of 7 and 20 days. Two normal birds were injected with culture and exudate, respectively, as a check on the infectivity of these agents. The reinjected birds were held under observation for 2 weeks and examined daily.

The initial injection was followed in all cases by a coryza which tended to persist for a longer time in the birds which had received exudate. Throughout the period of observation the birds which had recovered from the "bacillary" coryza showed no response to the injected exudate. In 2 cases the interval between recovery and the second injection had been only 7 days. The control bird developed a nasal discharge on the 2nd day. The fowl which had recovered from the "exudate" coryza likewise failed to respond to the second injection, in this case of the bacillus. In these birds the interval between recovery and reinjection had been 14 days or longer. The normal bird which had been injected with the bacillus showed a coryza on the 2nd day. A summary of these observations is presented in Table I.

The preceding protection tests on the first coryza (Coryza I) were carried out in the spring of 1932 and shortly after their completion the maintenance of the disease by bird to bird transfer was discontinued. In the fall attention was focused on a second coryza (Coryza II) originally obtained from a different source.[2] Protection tests were conducted with it and also with a third coryza (Coryza III) secured from the same poultry farm as the first, but a year later.

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[2] The natural case of this coryza was obtained by Dr. O. Seifried and Dr. C. Cain.

Coryza II was first produced in February, 1932, by the intranasal injection of normal fowl with exudate obtained from a naturally affected bird and up to July the disease was maintained by bird to bird passage. Both Rhode Island Reds and White Leghorns were used in the experimental production of the disease. It differed from Coryza I with respect to the onset and duration of symptoms. Following the injection of exudate, symptoms were regularly delayed until the 7th to the 14th day but thereafter persisted for 2 months or longer. Exudate filtered through N and V Berkefeld candles was tested and found to be innocuous. Several unsuccessful attempts to isolate the specific bacillus by filtration were also made. The disease was maintained during the summer by contact infection and in September exudate from these birds was injected intranasally in normal fowl with the finding that the incubation period of the disease had become reduced to 24 or 48 hours. The course of the coryza, however, was not altered and symptoms continued to persist for 2 months or longer. By the use of sealed plates of blood agar, the specific bacillus was readily isolated from the nasal exudate of these birds and regularly produced a coryza upon injection in normal fowl. The incubation period of the "bacillary" coryza was 24 to 48 hours, as in the case of the "exudate" coryza, but the course of the disease was much shorter, averaging 14 days in 20 cases.

TABLE I

Cross-Protection Tests with "Bacillary" and "Exudate" Forms of Coryza I

No. of bird	Material injected	Incubation period	Duration of symptoms	Period between recovery and second injection	Material injected	Result of injection
		days	*days*	*days*		
1	Exudate	2	18	19	Culture	Normal 14 days
2	"	2	4	17	"	" 14 "
3	"	2	17	14	"	" 14 "
4	"	2	11	16	"	" 14 "
Control						Coryza 2nd day
5	Culture	1	3	7	Exudate	Normal 14 days
6	"	2	5	9	"	" 14 "
7	"	2	12	7	"	" 14 "
8	"	1	4	20	"	" 14 "
Control					"	Coryza 2nd day

It was found that birds which had recovered from the "bacillary" and "exudate" types of Coryza II were resistant to reinfection with the bacillus and with exudate, respectively. Ten birds were employed in each experiment. 2 to 4 weeks after the initial coryza, produced in

It was apparent from the above experiment that recovery from the "bacillary" coryza was attended by a considerable degree of immunity to reinfection with the bacillus. Reciprocal protection tests were then carried out to determine whether birds which had recovered from the "bacillary" disease were immune to reinfection with exudate and *vice versa.*

Two groups of 4 birds, confined in individual cages in separate quarantine units, were employed in this experiment. 0.5 cc. amounts of 24 hours old horse blood agar cultures of the specific bacillus were injected into the palatine cleft of the birds in one group. 0.5 cc. amounts of exudate, withdrawn from several fowl previously infected with "exudate" coryza, were similarly injected in the birds of the second group. After recovery from the initial coryza the birds of the first group were injected with exudate and those of the second with cultures of the bacillus. The interval between the disappearance of symptoms and the second injection varied from bird to bird with extremes of 7 and 20 days. Two normal birds were injected with culture and exudate, respectively, as a check on the infectivity of these agents. The reinjected birds were held under observation for 2 weeks and examined daily.

The initial injection was followed in all cases by a coryza which tended to persist for a longer time in the birds which had received exudate. Throughout the period of observation the birds which had recovered from the "bacillary" coryza showed no response to the injected exudate. In 2 cases the interval between recovery and the second injection had been only 7 days. The control bird developed a nasal discharge on the 2nd day. The fowl which had recovered from the "exudate" coryza likewise failed to respond to the second injection, in this case of the bacillus. In these birds the interval between recovery and reinjection had been 14 days or longer. The normal bird which had been injected with the bacillus showed a coryza on the 2nd day. A summary of these observations is presented in Table I.

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a coryza of slow onset after passage through 4 susceptible birds. The experimental findings with Coryza II are presented in Table II.

Exudate from each of 10 recovered birds, which developed a coryza after the injection of exudate, was examined bacteriologically using sealed plates of horse blood agar. In all cases a culture was made shortly after the appearance of the nasal discharge and in some cases 2 or more additional cultures were made at later intervals. The specific bacillus was not isolated in a single instance from these plates.

TABLE II

Cross-Protection Tests with "Bacillary" Coryza II and Infectivity Tests with Exudate from the Reinjected Birds

Protection tests					Infectivity tests	
No. of bird	Incubation period of "bacillary" coryza	Duration of "bacillary" coryza	Interval between recovery and injection of exudate	Reaction of the recovered birds	No. of bird	Reaction of the susceptible birds
	days	*days*	*days*			
1	1	31	21	Coryza after 13 days	1-A	Normal 30 days
2	1	16	18	" " 21 "	2-A	" 30 "
3	1	21	22	" " 15 "	3-A	" 30 "
4	1	17	22	" " 13 "	4-A	" 30 "
5	1	18	34	" " 15 "	5-A	Coryza after 12 days
6	1	10	22	" " 10 "	6-A	" " 16 "
7	1	25	14	" " 18 "	7-A	" " 13 "
8	1	21	10	" " 15 "	8-A	" " 10 "
9	2	7	24	" " 17 "	9-A	" " 13 "
10	1	13	14	" " 14 "	10-A	" " 18 "
11	1	6	18	Normal 30 days		

The bacteria which developed on the plates were the usual organisms which inhabit the nasal mucosa of both normal and affected birds; no additional bacteria were encountered.

A bacteriological examination was also made of exudate removed on the 1st day of the coryza from Birds 5-A, 6-A, 8-A, and 9-A. Practically a pure growth of the specific bacillus was obtained on sealed plates streaked with exudate from Nos. 5-A, 8-A, and 9-A. It was not recovered, however, on the plate from No. 6-A. Exudate from each of one series by injection of the bacillus and in the other by exudate, had subsided, the birds of the first series were reinjected with the bacillus and those of the second series with exudate. The reinjected fowl were held under observation for a period of 30 days and during this time all of them remained normal. Susceptible birds injected with the same infecting agents developed a coryza on the 2nd day.

A reciprocal protection test was then carried out to determine whether birds which had recovered from the "bacillary" coryza were also resistant to reinfection with exudate. The outcome of this experiment showed clearly that the resistance acquired as the result of infection with the specific bacillus was at least quantitatively different from that acquired after recovery from the "exudate" coryza.

Eleven birds were employed in this experiment. 10 to 34 days after recovery from the initial coryza, produced by the injection of 0.5 cc. amounts of 24 hours old horse blood agar cultures of the specific bacillus, from Coryza II, they were injected with 0.5 cc. amounts of exudate, removed from the nasal passages of several fowl affected with "exudate" coryza. Prior to injection the exudate was rubbed up with a little bouillon in a glass tissue grinder and diluted to approximately 10 cc. with bouillon. A susceptible bird was also injected with 0.5 cc. of the same material. The reinjected fowl were held under observation for a period of 30 days and examined daily. Exudate was removed from each of the birds which developed a second coryza, shortly after symptoms appeared, and a 0.5 cc. portion injected into a normal fowl. The birds of this series were also examined daily over a period of 30 days.

Ten of the 11 birds which were injected with exudate, following recovery from the "bacillary" coryza, showed an inflammation of the nasal mucosa with a discharge from the nares. In each case, however, symptoms appeared only after a long incubation period which varied from 10 to 21 days. The eleventh bird remained normal throughout the period of observation. The control showed symptoms of coryza on the 2nd day. Six of the 10 birds which were injected with exudate, removed from the nares of the reinjected fowl, developed a coryza after an incubation period of 10 to 18 days. Four of the birds in this series remained normal during the period of observation. A repetition of the entire experiment with Coryza III gave essentially the same results. Exudate from 1 recovered bird, which had developed a second coryza following the injection of exudate, continued to produce coryza may, however, retain their particular characters for a considerable period of time.

Coryzas I and III were also produced experimentally by injection of the specific bacillus isolated from the nasal exudate but in both cases the duration of the disease was much shorter than that of the coryza produced by exudate. Protection tests were carried out to determine whether this discrepancy was due to the presence of a second infectious agent, acting independently of or in conjunction with the specific bacillus, or to some other factor.

The demonstration of reciprocal protection in the case of Coryza I was directly opposed to this view but the course of events with Coryza II, after its incubation period had become reduced, and with Coryza III seemed at first to favor it. In the case of the latter coryzas most of the birds which had recovered from the "bacillary" infection showed a nasal discharge following the injection of exudate. Subsequent observations, however, suggested another explanation; namely, that the degree of immunity produced by the cultivated bacilli was not sufficiently high, in most cases, to actually destroy the bacilli present in exudate but was sufficient to retard their development. There was reason to believe, moreover, that growth of the latter bacillus in the partially immune host was attended by a temporary change in certain of its characters. Exudate from some of the reinjected fowl was infective for normal birds but with the first passage and sometimes with several additional passages it produced a coryza of slow onset. Eventually, however, the exudate returned to its normal level of infectivity and for some unknown reason the isolation of the specific bacillus was most readily accomplished immediately preceding this change.

As a working hypothesis, it is suggested that the fluctuations observed in the onset and course of the coryzas from different sources and in the "bacillary" and "exudate" forms of the disease are referable to changes induced in the specific bacillus by unfavorable environmental conditions, in the one case by growth in a partially immune host and in the other by artificial cultivation. The existence of a second infectious agent is not definitely excluded but no evidence that would support a dual etiology has been forthcoming. The non-specific effect of secondary invaders, which are numerous in the injected exuthese 4 birds was also injected intranasally in normal fowl. The incubation period of the resulting coryza was 24 hours in each case. The specific bacillus was isolated from the fowl injected with exudate from No. 6-A. The 4 strains of the coryza organism which were isolated from the birds of this series were injected into normal fowl and in each case produced a coryza after an incubation period of 24 hours.

Protection tests were also carried out with a number of birds which had been exposed to Coryza II by direct contact. In this experiment 5 normal fowl were placed in the same pen with 5 birds affected with "bacillary" coryza and a similar number in contact with 5 affected with "exudate" coryza. In each series 4 of the exposed birds developed a coryza, those in contact with the "bacillary" cases after 6, 7, 7, and 8 days and those in contact with the "exudate" cases after 4, 5, 6, and 8 days. One bird in each series remained normal throughout the period of contact, which was continued until most of the birds had recovered. 2 weeks or more after recovery, 3 contact birds from each group were injected with the same agent to which they had been exposed. In both cases this number included 2 birds that had developed coryza as a result of exposure and the 1 unaffected bird. The injected fowl were held under observation for 30 days and during this time none of them showed symptoms of the disease.

DISCUSSION

The existence of several types of uncomplicated fowl coryza, differing from one another in the length of the incubation period and the course of the disease, is indicated by the preceding observations. Three such types were encountered; namely, Coryza I with a rapid onset and relatively short course, Coryza II with a slow onset and a prolonged course, and Coryza III with a rapid onset and a prolonged course. There is a suggestion, from the experimental study of the 3 types, that Coryza III is the basic form of the disease and that Coryzas I and II are variants which tend to revert to it with continued passage through susceptible fowl. Thus, Coryza III was obtained from naturally affected birds of the same flock from which Coryza I had been secured a year earlier. In the case of Coryza II a reduction in the originally prolonged incubation period occurred with the continued passage of exudate from bird to bird. The variant types of the coryza may, however, retain their particular characters for a considerable period of time.

Coryzas I and III were also produced experimentally by injection of the specific bacillus isolated from the nasal exudate but in both cases the duration of the disease was much shorter than that of the coryza produced by exudate. Protection tests were carried out to determine whether this discrepancy was due to the presence of a second infectious agent, acting independently of or in conjunction with the specific bacillus, or to some other factor.

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SUMMARY

Three types of an uncomplicated fowl coryza, differing in the onset and duration of symptoms, developed after the intranasal injection into normal birds of exudate from natural cases. Protection tests were carried out with 2 of the types in an attempt to explain why the "bacillary" disease regularly ran a shorter course than the "exudate" disease. Reciprocal protection was demonstrated in one case, but in the other the birds which had recovered from the "bacillary" disease were susceptible to reinfection with exudate. There was no indication, however, that a second infectious agent was present in the exudate, and the failure to cross-immunize was ascribed, rather, to a reduction in the immunizing properties of the specific bacillus induced by artificial cultivation.

It was also noted that the coryzas produced by exudate and bacilli, respectively, could be transmitted from infected birds to normal ones by direct contact. In both cases 1 bird out of 5 failed to contract coryza on exposure. These 2 birds were later injected with the respective agents to which they had been exposed and found to be resistant.

EXPERIMENTAL EPIDEMIOLOGY OF TUBERCULOSIS

THE EFFECT OF A PRIMARY INFECTION ON CONTACT TUBERCULOSIS IN RABBITS

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PLATE 16

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A study has been made of rabbits vaccinated with living tubercle bacilli of human type and subsequently exposed to tuberculosis under conditions simulating the natural modes of human contagion. In view of the present world-wide study of the prophylactic inoculation of human beings with BCG and other vaccines, the desirability of a method by which the safety and efficacy of a given procedure can be tested in small laboratory animals is obvious. The method employed in the present experiments can be used to throw light upon this and other problems in tuberculosis.

That conclusions drawn from inoculation experiments are not directly applicable to infection under natural conditions has been shown in the extensive experience of the last two decades with the vaccination of cattle. Although cattle treated with the bovovaccine of von Behring resisted for several months fatal doses of tubercle bacilli of bovine type introduced artificially, these animals nevertheless acquired tuberculosis when stabled for a year or more with cattle scattering tubercle bacilli (1). On the other hand it has been shown (2) that the laborious process of exposing experimental animals to tuberculosis by contact presents a much closer analogy to the disease as it occurs naturally in man.

The original observations of Robert Koch, showing that guinea pigs and rabbits acquire tuberculosis by contact with tuberculous animals, has found wide confirmation (3) in recent years. In the laboratory of The Phipps Institute, investigations on contact tuberculosis in the guinea pig (2) have clearly distinguished infection acquired by way of the alimentary, and by way of the respiratory tract, and showed that the route of infection depended on the relative intensity of exposure by one or the other channel (2).[1] As in man, the engrafting of tuberculosis

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[1] Lurie (2), 1930, page 769.

by the alimentary route inhibits the development of respiratory disease. Again as in man, alimentary tuberculosis in the guinea pig runs a more chronic course than the respiratory infection (2).[2] Furthermore the incidence of contact tuberculosis was greatly increased by crowding, a factor of great importance in the epidemiology of the disease among human beings. These points of similarity were offset, however, by the fact that in guinea pigs the incidence of tuberculosis is low when acquired by contact, especially when acquired by way of the respiratory tract.

In the present experiments, rabbits have been used. It was thought that in this animal the incidence of tuberculosis under the same experimental conditions might be higher than in the guinea pig. It has been shown (2)[3] that the determining factor in the effect of crowding upon the incidence of tuberculosis is the amount of bacilli available for contagion. In the rabbit the kidney is one of the favorite sites of tuberculosis, with frequent involvement of the medulla, and excretion of large numbers of tubercle bacilli in the urine. Moreover the great difference in the susceptibility of this animal to tubercle bacilli of human type on the one hand, and of bovine type on the other, provided a method by which a primary lesion produced by vaccination with the less virulent type could be definitely distinguished from a lesion acquired by contact with tuberculous animals. By this means, also, tuberculosis characteristic of white adults could be imitated.

It is generally admitted that adult type tuberculosis in the European races is an exogenous or endogenous reinfection following the primary infection of infancy and childhood. A localized, non-progressive infection with living human type bacilli in rabbits might serve experimentally, therefore, as a primary lesion comparable to that acquired by man during childhood. If rabbits so treated, and then exposed to cage mates infected with bovine type bacilli, were to develop progressive tuberculosis, the lesion could be identified by isolating the causative agent in pure culture and determining its type.

Preliminary experiments showed, as had been expected, that the incidence of contact tuberculosis in normal rabbits exposed to cage mates infected with bovine type bacilli was much greater than in guinea pigs similarly exposed. Rabbits exposed to cage mates infected with the human type bacillus did not acquire the disease. Accordingly the following experiment was begun.

Method

Metal cages were built, measuring 27 inches in width, 32 inches in depth, and 15 inches in height, and equipped with wire mesh doors. To increase the ventilation, a window, 6 by 8 inches, was cut out in the back of the cages 4 inches above

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[2] Lurie (2), 1930, page 753.

[3] Lurie (2), 1930, page 729.

the floor, and covered with wire mesh. Into each of 7 cages, 8 rabbits were placed upon a metal pan having an approximate area of 6 square feet. 4 rabbits were infected intravenously with 0.001 to 0.000,01 mg. of a highly virulent strain of bovine type tubercle bacilli, "Ravenel," and served as sources of contagion for the other 4 animals in the cage. 2 of the latter were normal half grown rabbits free from tuberculous infection. The other 2 were rabbits of similar age that about 2 months previously had been given a subcutaneous inoculation of 1.0 mg. of a virulent human type tubercle bacillus, No. P-15 B.

In these cages the normal and infected rabbits were exposed under identical conditions to infection both by way of the alimentary and respiratory tracts.

To determine whether normal and infected rabbits will acquire tuberculosis under conditions eliminating gross alimentary infection, another cage of the dimensions given above was divided through the middle by a vertical partition of galvanized iron soldered to the walls. A window, 25 inches in length and 8 inches in height, was cut into this partition 6 inches above the floor. This opening was covered with 1/16 inch wire mesh gauze soldered to its edges. 4 rabbits inoculated intravenously with 0.001 to 0.000,01 mg. Ravenel strain were placed upon a pan in one compartment of the cage. These served as sources of contagion for 4 rabbits placed in the other compartment, 2 normal, and 2 vaccinated with human type bacilli as described above. To increase the circulation of air from the sources of contagion to the contacts, a slit, 26 x 3 inches, was made in the solid metal door of each compartment, 4 inches from the floor in the door closing upon the sources of contagion, and 11 inches from the floor in the door closing upon the contacts. The air warmed by the sources of contagion rises upward, passes through the wire mesh screening into the compartment containing the contacts and passes out through the slit in the door of this compartment. This 8th cage was referred to as the "respiratory cage."

The pans on the floor of the cages were bedded with peat moss. Food in adequate amounts was placed upon them daily, consisting of hay, oats, and bread soaked in water, with fresh green vegetables twice a week. The cages were cleaned three times weekly on alternate days. In case of death of any animal from an intercurrent infection the cage was sterilized.

The population of these 8 cages was kept constant by replacing the dying animals, either the sources of contagion or the contacts, by similarly treated or normal animals.

The extent of tuberculosis in the inoculated sources of contagion was determined at their death. In some of these the urine from the bladder was stained for tubercle bacilli.

The experiment was continued for over 1 year. At the end of this time all the remaining contacts were killed and a careful autopsy was performed on each of them. A diagnosis of tuberculosis in "normal contacts" was invariably confirmed by numerous smears for acid-fast bacilli. In 1 animal the virulence of a pure culture obtained from the lung was the same as that of the Ravenel strain used to inoculate the sources of contagion. The tuberculous lesions of animals that had a primary infection with human type tubercle bacilli before exposure was begun, were cultured for tubercle bacilli after sodium hydroxide treatment. Their virulence was determined by inoculating 2 rabbits intravenously with 0.01 mg. of these cultures or of cultures obtained from other animals, rabbits or guinea pigs, that had been injected with the lesions of the contact animal. In this way the lesions found in these contacts could be definitely ascribed either to exogenous reinfection with the bovine type bacillus or to endogenous spread from the primary lesion caused by the subcutaneous inoculation with the human type bacillus. In some rabbits both the residual primary lesion in the subcutaneous tissues and the lesion in the lungs were cultured and the virulence of each culture determined. In some vaccinated contacts the type of the bacillus in the tuberculous lesions was determined by the direct intraperitoneal inoculation into rabbits; if death from massive generalized tuberculosis and tuberculous peritonitis resulted in about 2 months the lesion was considered to have been caused by the bovine type bacillus.

Sections for microscopic study were prepared from the lesions of all contacts that developed tuberculosis.

RESULTS

The Primary Lesion of Vaccination

Before considering the main results of the experiment it is of interest to follow the course of the primary infection in the vaccinated contacts.

About 1 week after the subcutaneous inoculation of 1.0 to 0.01 mg. of the human type strain, P-1-5 B, a subcutaneous nodule developed, varying in diameter from 9 to 19 mm. The skin overlying this nodule was usually not adherent. The nodule gradually enlarged and the draining lymph nodes became affected between the 2nd and 3rd week after inoculation. The nodule then became softened and fluctuating. In rare instances the lesion ulcerated through the skin, and with the discharge of the caseous pus the ulcer healed. Usually the subcutaneous lesions became thoroughly encapsulated in fibrous tissue and remained dormant, although they contained living virulent tubercle bacilli of the human type even 1 year after inoculation. In other cases they resolved and left no trace behind, or left a flat area of brownish pigmented tissue consisting of regressive epithelioid cells (4). In one instance the lesion became calcified. In most cases the lesion was completely localized and the affection of the lymph nodes was transitory. In other instances, small numbers of tubercles with inconspicuous caseous centers were found in the lung and kidney. Rarely one or several encapsulated pus pockets swarming with living tubercle bacilli were found i [illegible]. In 1 rabbit there was a lesion in the ileocec [illegible] containing v [illegible] rcle bacilli. In all these lesions the bacilli we [illegible] an type.

Contact Tuberculosis in Vaccinated and Normal Rabbits: Protocols

In *Cage 1*, 2 normal rabbits and 4 vaccinated rabbits were exposed to tuberculous rabbits. *Normal Rabbits.*—N-1-9 was killed after 373 days of exposure. The appendix, ileocecal region, and mesenteric nodes were tuberculous. There was an extensive tuberculosis of the pleura with pearl formation. The omentum was tuberculous. Large encapsulated pus pockets with a moderate number of discrete tubercles were found in both lungs. The tracheobronchial lymph nodes were not affected. The right kidney was extensively, and the left slightly involved. N-1-1 was killed after 430 days of exposure to a total of 26 tuberculous rabbits. No gross or microscopic tuberculosis could be found, nor could tuberculosis be demonstrated by guinea pig inoculation from non-specific lesions in the lung of this rabbit.

Vaccinated Rabbits.—I-5 and I-4 died with tuberculosis after an exposure of 355 and 290 days respectively. In I-5 there was no primary lesion of vaccination; in I-4 there was a fibrous scar. I-5 showed tuberculosis of the appendix with advanced fibrosis microscopically. The mesenteric nodes were not grossly affected. Caseous nodes were found in the pleura. Both lungs showed a moderate number of discrete tubercles, some 10 mm. in diameter, some firmly encapsulated with crumbly centers. There was a small number of tubercles in the cortex of each kidney. The cause of death was an intestinal infection. I-4 died with tuberculosis of the appendix and mesenteric nodes, and an extensive discrete and conglomerate tuberculosis consolidating both lungs. The tracheobronchial nodes were slightly affected. There were caseous nodes in mediastinum and pericardium. The kidneys and bone marrow showed an extensive tuberculosis. Intraperitoneal injection of the lesions from the lung and pleura of each of these contacts into 2 normal rabbits caused fatal generalized tuberculosis in 34 to 71 days. I-5-0 and I-5-5, exposed for 139 and 100 days respectively, showed no tuberculosis except at the site of vaccination, which contained tubercle bacilli virulent for guinea pigs.

In *Cage 2*, 5 normal and 4 vaccinated rabbits were exposed. *Normal Rabbits.*—N-5-2 and N-7-0 each developed a single pulmonary tubercle 7 and 5 mm. in diameter respectively after 92 and 97 days of exposure. Both were extensively caseated and contained numerous tubercle bacilli. The tracheobronchial nodes were enlarged in both contacts and in N-7-0 they contained tubercle bacilli. N-6 died after 210 days of exposure with tuberculosis of the cervical nodes and appendix. The mesenteric nodes were free of tuberculosis. The lungs were consolidated by a massive caseous pneumonia. The tracheobronchial nodes were slightly affected. There was tuberculosis of the pleura and kidney and extensive tuberculosis of the bone marrow. N-1-0 and N-7-5 showed no tuberculosis after an exposure of 320 and 95 days respectively.

Vaccinated Rabbits.—None acquired tuberculosis. I-1-1 and I-5-4 exposed for 310 and 128 days respectively, showed no tuberculosis except at the site of vaccination. I-1-3 and I-5-3 exposed for 243 and 129 days respectively, showed in addition slight pulmonary lesions. These lesions caused slight non-progressive tuberculosis 67 and 61 days after intraperitoneal inoculation into normal rabbits.

In *Cage 3*, 4 normal and 3 vaccinated rabbits were exposed. *Normal Rabbits.*—N-6-6 developed a single centrally caseated tubercle 6 mm. in diameter containing numerous tubercle bacilli in the lower lobe of the left lung after 109 days of exposure. The tracheobronchial nodes were enlarged. N-8 died after 194 days of exposure to a total of 12 tuberculous rabbits with consolidation of both lungs by extensively caseated discrete tubercles, caseous tuberculosis of the tracheobronchial nodes, but no gross tuberculosis of appendix, mesenteric or cervical nodes. The pleura and mediastinum were tuberculous with pearl formation. Miliary tuberculosis was found in the omentum. The kidneys and bone marrow were moderately tuberculous. The disease of respiratory origin in this contact may be contrasted with the tuberculosis acquired by the enteric route by N-1-8, which was killed after 375 days of exposure to a total of 23 tuberculous rabbits. There was extensive tuberculosis with ulceration through the mucosa in the appendix and ileocecal region, extensive caseous tuberculosis with liquefaction of mesenteric nodes, miliary tuberculosis of omentum, extensive tuberculosis of pleura and pericardium with pearl formation, and widespread discrete and conglomerate tuberculosis of the lungs with extensive caseation. The tracheobronchial nodes were not affected. The kidneys were slightly tuberculous. N-4-7 died after 136 days of exposure, of an intestinal infection. There was no gross tuberculosis.

Vaccinated Rabbits.—I-3 and I-6 developed an enteric infection limited to the appendix, ileocecal region, and mesenteric nodes after an exposure of 383 and 242 days respectively. Pure cultures obtained from these lesions either directly or after preliminary passage through a guinea pig caused fatal generalized tuberculosis in normal rabbits in 24 to 43 days. Both contacts had residual lesions at the site of vaccination. I-4-0 was killed after 191 days of exposure. There was an isolated caseous focus 3 mm. in diameter in the ileocecal region. The mesenteric nodes were not affected. An isolated caseated tubercle 8 mm. in diameter was found in the lower lobe of the right lung. The tracheobronchial lymph nodes were free of tuberculosis. There was extensive tuberculosis of the pleura with pearl formation. At the site of subcutaneous inoculation of 1.0 mg. of a human strain of tubercle bacillus, A-1-D, there was an encapsulated nodule 15 mm. in diameter. A pure culture obtained from this primary lesion caused slight non-progressive tuberculosis in rabbits; a pure culture isolated from the lung and pleura caused fatal tuberculosis in 34 and 36 days after intravenous inoculation of 0.01 mg. into rabbits.

In *Cage 4*, 4 normal and 4 vaccinated rabbits were exposed. *Normal Rabbits.*—N-7-1 was killed after 98 days of exposure. There was an isolated focus 2 mm. in diameter in the ileocecal region and one in the appendix, 1 mm. in diameter. Innumerable tubercle bacilli were found in the smear of the lesion in the ileocecal region and also in the tuberculous mesenteric nodes. N-9 and N-4-0 died of massive, caseous pneumonia with caseation of the tracheobronchial nodes after 194 and 155 days of exposure respectively. Both had moderate to extensive tuberculosis of the kidneys, and N-4-0 had tuberculosis of cervical and mesenteric nodes and a specific lesion in the right testicle. N-6-1 was killed after 140 days of exposure. There were two extensively caseated tubercles 7 and 4 mm. in diameter in the upper lobe of the left lung. The tracheobronchial nodes were tuberculous. There was beginning tuberculosis of the pleura with pearl formation.

Vaccinated Rabbits.—I-3-9, killed after 182 days of exposure, is to be contrasted with N-6-1. There were two tubercles in the lower lobe of the right lung, 12 and 5 mm. in largest diameter, with discrete foci of caseation. The tracheobronchial nodes were not affected. There was a moderate tuberculosis of the pleura with pearl formation. From the encapsulated subcutaneous abscess at the site of vaccination a pure culture of tubercle bacilli of the human type was isolated. From the pulmonary lesion a pure culture of the bovine type was obtained after preliminary passage through a rabbit. An essentially similar lesion was found in the lung of I-6A, killed after 56 days of exposure, and a pure culture of the bovine type was isolated. I-1-7 and I-8 died after 195 and 375 days of exposure respectively. Both showed tuberculosis of the appendix and mesenteric nodes, and extensive tuberculosis of the pleura and mediastinum, with, in I-1-7, pearl formation and involvement of the pericardium. There was an extensive discrete and conglomerate tuberculosis of both lungs in these contacts, with involvement of the tracheobronchial nodes in I-1-7. The tuberculosis of the kidneys was extensive in both cases, with excavation in I-8. This rabbit also showed tuberculosis of the uterine horns, trachea, and knee joint. The cervical nodes and the entire lymphatic system of I-1-7 were extensively affected. The primary lesion of vaccination was calcified in I-8, and in I-1-7 there was a subcutaneous encapsulated abscess containing tubercle bacilli. A pure culture obtained from the lung and pleura of I-8 caused fatal tuberculosis in rabbits in 32 and 37 days. The pulmonary lesion of I-1-7 injected intraperitoneally into 2 rabbits caused fatal tuberculosis in 35 and 44 days.

In *Cage 5*, 3 normal and 4 vaccinated rabbits were exposed. *Normal Rabbits.*—None of the normal rabbits, N-2-3, N-4-1, and N-1-6, exposed for 53, 307, and 432 days respectively developed tuberculosis, although N-1-6 was exposed to a total of 23 tuberculous rabbits.

Vaccinated Rabbits.—I-7 died of an unknown cause after 270 days of exposure, having a few discrete caseous tubercles in both lungs. A rabbit injected intraperitoneally with these lesions had a single pulmonary tubercle when killed in 77 days. I-2-6 died of pulmonary congestion and edema after 217 days of exposure. There were no tuberculous lesions anywhere in the body. I-9 was killed after 56 days of exposure and an encapsulated lesion with central softening 10 mm. in diameter, was found in the lower lobe of the right lung. The tracheobronchial nodes were not affected. A pure culture obtained from the lesion after preliminary passage through a rabbit caused fatal tuberculosis in 27 days in 1 rabbit. I-10 was killed after 437 days of exposure. There were irregular foci in the appendix and several nodules in the mediastinum, one with caseation. The mesenteric nodes were free of tuberculosis. Pure cultures obtained from the appendix and the normal rabbits. These 3 contacts had residual primary lesions of vaccination, those in I-1-8 and I-5-2 containing tubercle bacilli.

Incidence of Contact Tuberculosis

In the above protocols are given the most significant data for each of the 30 normal and 30 vaccinated contacts used in these experiments. As is seen there, tuberculosis was acquired by some of the contacts, either the normal, the vaccinated, or both, in each of the 8 cages. The incidence and course of the acquired disease was not apparently different in the respiratory cage, No. 8, in which the contacts were separated from the sources of contagion by a fine, wire mesh screen, and in the remaining 7 cages, in which the animals were not separated. All the contacts are therefore considered together.

In Table I the significant data and conclusions relative to each of these rabbits are summarized. The contacts are listed in the order of increasing duration of exposure. To facilitate reference to the protocols the number of the cage in which each contact was exposed is given.

Incidence in the Vaccinated as Compared with the Normal Contacts. —It can be seen that out of a total of 30 normal rabbits exposed for periods varying from 53 to 432 days, with an average exposure of 200, and a mean exposure of 155 to 163 days, 22 or 73.3 per cent developed tuberculosis. Out of a total of 30 vaccinated rabbits exposed for periods varying from 56 to 437 days, with an average exposure of 218 and a mean exposure of 196 to 217 days, 18 or 60 per cent acquired tuberculosis. That the tuberculous lesions in these 18 rabbits were caused by bovine type bacilli was demonstrated by the virulence for rabbits of pure cultures isolated from them or by fatal generalized tuberculosis resulting in from 24 to 47 days, and in one instance in 71 days, after the direct intraperitoneal inoculation of these lesions into other rabbits. In the remaining 12 rabbits, either there were no lesions beyond the site of vaccination or there were tuberculous lesions in other parts of the body caused by endogenous spread from the primary lesion, as shown by the recovery from them of tubercle bacilli of human type. The incidence of acquired tuberculosis among these rabbits was thus definitely less, 27 per cent of normal rabbits, and 40 per cent of the vaccinated rabbits having escaped infection.

TABLE I

The Incidence and Extent of Acquired Tuberculosis in Normal and Vaccinated Rabbits

Normal						Vaccinated					
Rabbit No.	Cage No.	Duration of exposure	Extent of tuberculosis	Fatal tuberculosis	Route of infection	Rabbit No.	Cage No.	Duration of exposure	Extent of tuberculosis	Fatal tuberculosis	Route of infection
		days						*days*			
N-2-3	5	53	0			I-6A	4	56	+		Resp.
N-7-8	6	59	0			I-9	5	56	+		Resp.
N-5-2	2	92	+		Resp.*	I-6-1	6	56	0		
N-7-5	2	95	0			I-5-7	7	84	+		Resp.
N-7-0	2	97	+		Resp.	I-1	7	90	0		
N-7-1	4	98	+		Ent	I-3-1	8	97	0		
N-7-2	7	98	++++		Resp.	I-5-5	1	100	0		
N-6-6	3	109	+		Resp.	I-5-2	8	126	0		
N-6-5	8	122	+		Resp.	I-5-4	2	128	0		
N-4-6	7	122	+++	†	Resp.	I-5-3	2	129	0		
N-4-2	6	125	++++	†	Al., Resp.	I-5-0	1	139	0		
N-4-7	3	136	0			I-3-9	4	182	++		Resp.
N-6-1	4	140	++		Resp.	I-4-0	3	191	++		Resp., Ent.
N-6-2	6	144	+		Resp.	I-1-7	4	195	+++	†	Al.
N-4-0	4	155	++++	†	Al., Resp.	I-3-2	7	196	+++	†	Resp.
N-1-4	6	163	+++	†	Ent., Resp.	I-2-6	5	217	0		
N-9	4	194	++++	†	Resp.	I-2	6	240	+++	†	Ent.
N-8	3	194	+++	†	Resp.	I-6	3	242	+		Ent.
N-3	7	203	++++	†	Resp.	I-1-3	2	243	0		
N-6	2	210	++++	†	Al.	I-7	5	270	0		
N-4-8	6	218	++++	†	Resp.	I-2-7	6	271	++		Ent.
N-4-5	7	274	++++	†	Resp.	I-4	1	290	+++	†	Ent.
N-4-1	5	307	0			I-1-1	2	310	0		
N-1-5	8	317	++++	†	Resp.	I-1-8	8	342	+++	†	Resp.
N-1-0	2	320	0			I-5	1	355	++		Ent.
N-2-2	8	354	++		Ent.	I-1-5	7	362	++++	†	Resp.
N-1-9	1	373	++		Ent.	I-8	4	375	+++	†	Ent.
N-1-8	3	375	+++		Ent.	I-1-4	6	381	++		Resp.
N-1-1	1	430	0			I-3	3	383	+		Ent.
N-1-6	5	432	0			I-1-0	5	437	++		Ent.

* Resp. = respiratory. Ent. = enteric. Al. = alimentary.

Incidence in Relation to Length of Exposure.—Unfortunately the tuberculin reaction in the skin of rabbits is not well marked, and x-ray examination of the lungs revealed only moderately advanced lesions. Hence the time of inception and the exact duration of the disease could not be determined. Still sufficient numbers of animals either were killed or died at different intervals of time so that a definite relation could be observed between the duration of exposure and the incidence of contact tuberculosis. If a rabbit failed to develop tuberculosis at the end of a certain interval it was supposed that it had not had the disease at a previous interval. For example, Rabbits N-4-1, N-4-0, N-1-1, and N-1-6, showing no evidence of tuberculosis when they were killed after 307 to 432 days of exposure, presumably had none after 100 or after 200 days of exposure, and in Table II they are included in each of the three intervals.

As can be seen from Table II, 33 per cent of normal rabbits acquired tuberculosis within the first 100 days of exposure. 66 per cent of normal rabbits acquired tuberculosis when exposed from 101 to 200 days. Further exposure of normal rabbits did not increase the incidence of the disease. Among the vaccinated animals 20 per cent acquired tuberculosis within the first 100 days of exposure. Of vaccinated rabbits exposed for from 101 to 200 days 33 per cent developed tuberculosis as compared with twice that percentage of normal rabbits similarly exposed. However further exposure of the vaccinated rabbits resulted in a conspicuous increase in the incidence of the disease. It is noteworthy that the total incidence of tuberculosis among normal contacts exposed for a period up to 200 days was 63.6 per cent whereas only about half as many vaccinated contacts, 36.8 per cent, acquired the disease.

Incidence in Relation to the Number of Sources of Contagion.—It is evident that the length of exposure would be an exact measure of the quantity of tubercle bacilli to which the contacts were exposed if the elimination of bacilli were constant throughout the disease. Since, however, they are expelled chiefly in the later stages, it is significant that the incidence of acquired tuberculosis increased with an increase in the number of animals to which the contacts were exposed, as the sources of contagion that died of tuberculosis were replaced.

Vaccinated rabbits escaped infection after exposure to increasing numbers of tuberculous rabbits to a greater degree than did normal animals, but again only up to a certain point. For although 3 normal rabbits, N-1-0, N-1-1, and N-1-6, escaped infection completely after having been exposed to a total of from 20 to 26 tuberculous rabbits during a period of 320 to 432 days, none of the 7 vaccinated contacts listed last in Table I escaped exogenous infection after having been exposed to a total of from 21 to 23 rabbits for a period of 342 to 437 days. Thus the contrast observed between the vaccinated and the normal contacts in the incidence of contagion in relation to length of exposure holds equally in relation to the number of tuberculous rabbits to which the contacts were exposed.

TABLE II

The Incidence of Contact Tuberculosis in Normal and Vaccinated Rabbits in Relation to the Duration of Exposure

	Duration of exposure									
	7 wks. to 100 days		101 to 200 days		7 wks. to 200 days		201 to 437 days		7 wks. to 437 days	
	Normal	Vaccinated	Normal	Vaccinated	Normal	Vaccinated	Normal	Vaccinated	Normal	Vaccinated
No. exposed	12	15	15	12	22	19	12	15	30	30
No. developing tuberculosis	4	3	10	4	14	7	8	11	22	18
Percentage incidence of tuberculosis	33.3	20.0	66.6	33.3	63.6	36.8	66.6	73.3	73.3	60.0

Incidence in Relation to the Primary Lesion.—No constant relationship was noted between the extent of the primary lesion of vaccination and the incidence of acquired tuberculosis. Some vaccinated rabbits developed fatal tuberculosis in the presence of extensive local primary lesions containing living tubercle bacilli, *e.g.* I-2, I-3-2, and I-1-7, and other rabbits with slight or no residual primary lesions resisted infection for a long time, *e.g.* I-1-1, I-1-3, and I-2-6. Nor was there any constant relationship between the incidence of the disease and the interval elapsing between vaccination and exposure; for example, I-3-2 died from generalized tuberculosis after having been exposed, 35 days after vaccination, for 196 days, and Rabbit I-4-0 developed only slight tuberculosis after having been exposed, 114 days after vaccination, for 191 days.

Portal of Entry and the Acquired Primary Lesion

The route of infection in each of the 22 normal and 18 vaccinated contacts that acquired tuberculosis is given in Table I; the detailed facts upon which these conclusions were based are recorded in the protocols. In 18 of the 40 cases the location of the acquired primary lesion could be determined with certainty. In the remaining 22 cases the diagnosis of a respiratory infection was based upon observation of tuberculosis of the lungs with or without tuberculosis of the tracheobronchial lymph nodes, in the absence of any significant lesions in the intestines, the mesenteric and cervical lymph nodes. When tuberculosis of the intestines was found with or without tuberculosis of the mesenteric lymph nodes, in the absence of significant tuberculosis of the tracheobronchial nodes, the diagnosis of an enteric infection was made. If the cervical lymph nodes were extensively affected the infection was considered to be of alimentary origin by way of the pharynx. When lesions were found simultaneously in the tracheobronchial and mesenteric lymph nodes the diagnosis of a combined alimentary and respiratory infection was made.

TABLE III

The Portal of Entry in Normal and Vaccinated Rabbits with Tuberculosis Acquired by Contact

Respiratory only				Respiratory and alimentary				Alimentary only			
Normal		Vaccinated		Normal		Vaccinated		Normal		Vaccinated	
No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
14	63.6	8	44.4	3	13.6	1	5.5	5	22.7	9	50.0

Table III shows the marked difference in the route of acquired infection in the normal as compared with the vaccinated animals. In the normal animals the respiratory route was involved either alone or together with the alimentary tract in 17 out of the 22 cases, or in 77.2 per cent. In the vaccinated animal the alimentary route of infection was involved alone in 9 out of the 18 cases or in 50 per cent. It is noteworthy that although 19 normal rabbits had free access to alimentary contagion, being exposed in cages without the wire screen partition, nevertheless 12, or 63 per cent, acquired a respiratory infection.

In the rabbits in which the seat of the primary acquired lesion could be determined with certainty the distribution was as follows: In 11 of these the lesion was in the lung, 9 showing single, almost completely caseated tubercles, ranging in size from 4 to 12 mm. These were found in all parts of both lungs but most frequently in the lower lobe of the right lung. Each of 2 rabbits had 2 primary lesions in the lungs. These lesions in previously normal rabbits were associated with a marked enlargement and caseation of the tracheobronchial lymph nodes. 1 vaccinated rabbit had a single primary lesion in the lung and another in the ileocecal region, with no involvement of either the tracheobronchial or mesenteric lymph nodes. 5 rabbits had multiple primary lesions in the appendix and ileocecal region. 1 vaccinated rabbit, I-3, showed tuberculosis of the mesenteric nodes without any macroscopic lesions in the intestines. Thus the primary lesion acquired by the respiratory route was usually single in contrast to the multiple primary lesions acquired by the intestinal route.

Extent and Character of the Acquired Disease

A glance at Table I will show that the acquired disease was much more extensive in the previously normal animals than in the vaccinated animals. This appeared not only in the extent of involvement of the most susceptible organ, the lung, in which massive consolidation occurred 9 times in the normal contacts and only once in the vaccinated animals, but also in the dissemination to various other organs. Thus the kidney was affected in 14 and the bone marrow in 4 of the 22 normal contacts that acquired tuberculosis, while the kidney was affected in 7 and the bone marrow in 1 of the 18 vaccinated contacts that acquired tuberculosis.

Usually the acquired primary lesion in the lung was completely caseated in the normal contacts, and less extensively caseated in the vaccinated rabbits. Irrespective of the portal of entry the acquired tuberculosis was chiefly seated in the lung, and it was the extent of the disease in this organ that killed the contacts, whether they were normal or vaccinated, whether they acquired the infection by the alimentary or respiratory tracts. In the normal contacts the disease was characterized by a massive caseous pneumonia, as observed in 9 out of the 11 normal contacts that died with tuberculosis (Fig. 1). In the vaccinated animals the pulmonary lesion was usually a disseminated discrete tuberculosis (Fig. 2). In only 2 vaccinated contacts, I-1-5 and I-1-8, was there caseous pneumonia. It is noteworthy that both these rabbits had been given the smallest vaccinating dose and that in I-1-5 no lesion was observed under the skin before exposure was begun, nor was any subcutaneous lesion of primary infection found at autopsy.

In normal animals the lymph nodes draining the site of the acquired primary infection, whether in the respiratory or in the alimentary tract, were affected in each of the 22 that developed the disease. In vaccinated animals, on the contrary, these nodes were involved in only 6 out of 18 rabbits. It is noteworthy that in only 1 of the 8 vaccinated animals that acquired a respiratory infection were the tracheobronchial lymph nodes affected; namely, in I-1-5 cited above, which behaved in other respects also like a previously normal animal. In 5 out of the 9 instances of contact tuberculosis acquired by vaccinated rabbits by the enteric route the mesenteric lymph nodes were tuberculous. This is to be correlated with the observation that the acquired primary lesion in the lung is usually single whereas the primary lesion in the intestines is usually multiple.

It has been noted that the intestines were more frequently the portal of entry in the vaccinated than in the normal contacts. In accord with this is the observation that the ileocecal region and the appendix in vaccinated rabbits were more frequently diseased than in the normal contacts. In normal contacts significant tuberculosis of the intestines and mesenteric lymph nodes was rarely found in the presence of tuberculosis of the lung and tracheobronchial lymph nodes, although miliary tubercles were sometimes found in the intestines in association with a generally disseminated disease.

The route of infection influenced the course of the disease. This is clearly seen in the normal contacts that survived exposure for the longest period, such as N-2-2, N-1-9, and N-1-8. The extent of the disease acquired by the enteric route in these rabbits was much less after an exposure of 354 to 375 days than was the extent of tuberculosis of respiratory origin in similar contacts that died after an exposure as short as 122 days. In man, too, tuberculosis of alimentary origin is definitely more chronic in nature than the respiratory disease (5).

A lesion that is rarely seen in rabbits artificially inoculated with bovine type tubercle bacilli was found in the majority of contacts both normal and vaccinated. This is a tuberculosis of the pleura, which frequently simulates very closely the perlsucht of cattle, forming nodules, or pearls, which are often extensively caseated and often suspended by pedicles from the parietal or visceral pleura. When pleural involvement does occur in inoculated rabbits, it is seen only after a long continued chronic disease. In rabbits accquiring the disease by contact this lesion occurs soon after the primary lesion is established, whether in the lung or in the intestinal tract. The affection of the pleura may at times be extensive, compressing the lungs in the thoracic cavity. A further distinction of the natural from the artificial disease is the absence of acquired tuberculosis in the liver and spleen, organs that are frequently affected after artificial inoculation. In only 1 of the 40 contacts that acquired tuberculosis, N-2-2, were there a few tubercles in the spleen.

Microscopically the lesion in the lung of previously normal animals is characterized by widespread diffuse pneumonic and interstitial accumulations of large mononuclear and young epithelioid cells. These soon undergo massive caseation, leaving a few intact mature epithelioid cells. Bacilli are found in tremendous numbers during the earlier stages of the process. Mononuclears continue to accumulate about the advancing part of the lesion, and mitosis of these cells is unabated. The unhindered extension of the caseous process leads to hemorrhage and ulceration into the bronchi, resulting in rapid spread of the lesion to uninvolved areas of the lung. Discrete, mature, epithelioid cell tubercles are rare. There is little or no accumulation of lymphocytes about the tuberculous foci, and fibrous tissue formation is abortive, soon succumbing to the advancing caseous process. Vast areas of caseous tissue result, in which the outlines of the original alveolar walls indicate the essentially pneumonic origin of the caseous process.

In vaccinated animals discrete tubercles are formed, characterized by abundant intact epithelioid and giant cells. Tubercle bacilli are found in very small numbers. Caseation is much less extensive. Lymphocytes accumulate about the tubercles, and there is pronounced formation of granulation tissue. Many of the tubercles are resolving. However, despite this definitely healing process, characteristic of reinfection (6), the older caseous foci undergo softening, and tremendous numbers of tubercle bacilli accumulate in these areas, whence they invade the circulation and cause the slow extension of the disease, chiefly in the lung, kidney, and pleura.

Mortality

Out of the 30 normal contacts 11 died of tuberculosis after an average duration of exposure of 198 days, with a range of from 122 to 317 days. Out of 30 vaccinated contacts, 7 died of tuberculosis after an average exposure of 286 days, with a range of from 195 to 375 days. Vaccinated rabbits with fatal tuberculosis survived 88 days, or nearly 3 months, longer than the non-vaccinated contacts that died with the disease. The mortality from tuberculosis in the vaccinated contacts was therefore 13.3 per cent less than among the normal contacts. Thus the difference between the two groups in mortality is of the same magnitude as in the incidence of the disease.

DISCUSSION

Experiments are described in which 73 per cent of 30 normal rabbits exposed for about 1 year to cage mates infected with the bovine type tubercle bacillus acquired either a respiratory or an alimentary infection, which was fatal in 50 per cent of the cases. The effect upon contact tuberculosis of a localized non-progressive infection previously induced by vaccination with living tubercle bacilli of the human type, was studied in 30 rabbits exposed under identical conditions at the same time and in the same cages with the normal animals. In the large majority of instances the human type bacilli persisted with their characteristic virulence at the site of inoculation throughout the period of exposure. It was found that vaccination reduced the incidence and the mortality of the exogenously acquired tuberculosis, affected the route of infection, changed the extent and pathological character of the disease, and retarded its progress.

It was noted that the incidence in normal animals increased with increasing length of exposure during the first 200 days, and that further exposure did not increase the incidence of the disease among them. Essentially the same observation was made in a previous study of air-borne infection in guinea pigs, in which the incidence of acquired tuberculosis increased with the duration of exposure up to 2 years, but did not increase beyond that period (2).[4] This may be attributed to factors in natural resistance (7). On the other hand although it is evident that the vaccinated animals were protected during the first 200 days of exposure, for only 36.8 per cent of them developed tuberculosis as compared with 63.3 per cent of the normal animals, further exposure markedly increased the incidence of the disease in this group.

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[4] Lurie (2), 1930, page 743.

This might be thought of as due to the retarding effect of vaccination. However, the incidence of tuberculosis among the vaccinated animals exposed for from 201 to 437 days was actually greater than among the normal animals similarly exposed. 8 normal and 8 vaccinated rabbits survived this period, but of these, 4 normal rabbits completely escaped infection after an exposure of from 300 to 432 days, whereas only 1 vaccinated rabbit escaped infection after exposure of similar duration.

In this connection it is noteworthy that one or more fatal cases of contact tuberculosis occurred in each of the 8 cages except one. This was the only cage in which the normal animals escaped infection. However 2 vaccinated rabbits in this cage acquired a slight disease, although they were exposed for a shorter period than the normal contacts. There is no reason to suppose that natural resistance, which saved the normal rabbits from infection, should not also have been operative in the vaccinated animals, especially since there is ample evidence that vaccination gave considerable added protection. It is unprofitable to speculate as to the cause of this apparently deleterious effect that the vaccination seemed to exert on some contacts. This observation must first be confirmed and then studied further.

Although many present day investigators consider adult type pulmonary tuberculosis as usually due to exogenous reinfection, and although in this country work at The Phipps Institute (8) has produced important clinical evidence for this view, there are still eminent pathologists, such as Huebschmann (9) and Selter (10), who with von Behring, Römer, and Ranke consider exogenous reinfection of secondary importance as compared with endogenous metastatic tuberculosis. They base their view on the fundamental experiments of Römer (11) showing that guinea pigs that harbor a tuberculous infection are completely protected against small doses of tubercle bacilli introduced from without. Since the work of Chaussé (12), recently confirmed by Lange (13), showed that only minimal doses of bacilli can come into play in natural contagion, it is maintained that the primary lesion of childhood protects adults against the small numbers of bacilli that may penetrate into the tissues by way of exogenous infection. Furthermore, in a small number of experiments on guinea pigs, Römer found that the tuberculous animals resisted contact infection (14).

It has been noted that the primary lesion produced by vaccination had the essential characters of the primary focus of childhood, and in the majority of cases contained bacilli that retained their virulence when cultured. These lesions did. in fact, confer considerable [illegible] tection against infection. Nevertheless 60 per cent of the vaccinated animals acquired tuberculosis. Their disease was unequivocally shown to be of exogenous origin, for both the human type bacillus of the primary lesion, and the bovine type bacillus of reinfection could be isolated in pure culture from the same rabbit. Experimental proof is thus given that exogenous reinfection, acquired by the respiratory or the alimentary route, occurs in animals harboring a primary lesion. Römer's results with cattle are not conclusive in this respect, because the lesions due to vaccination were ephemeral, and in the large majority of instances they appeared completely healed at autopsy, and the human type tubercle bacilli previously contained in them had disappeared (15).

In applying these observations to adult type tuberculosis of man, it must be pointed out that the experimental animals were undoubtedly exposed to larger quantities of bacilli than are available to human beings under natural conditions. Nevertheless the mode of infection was in general the same; that is, continuous, prolonged, intimate contact with a source or sources of contagion, intensified by crowding. Moreover the disease acquired by these rabbits had many important points of similarity to tuberculous disease in white adults. Both are characterized by the failure of development of tuberculosis of the lymph nodes draining the portal of entry, the rarity of spreading lesions of caseous bronchopneumonia, and the limitation of hematogenous spread. On the other hand, although the disease was chiefly pulmonary, extensive cavity formation did not occur in these rabbits. It is impossible to say with certainty that the primary lesion in the vaccinated rabbits caused by the bacillus of the human type affords them a protection against the bovine type bacillus comparable to that afforded man by lesions acquired in childhood against reinfection with the human type bacillus. However there are rare but authentic instances in man of both the human and bovine type bacillus in the same person (16) and some parallel is presented in recent reports by many observers of bovine type tubercle bacilli isolated from human beings with pulmonary tuberculosis of reinfection, that is, of the adult type (17).

It has been observed in normal animals that the disease acquired by the respiratory route was more quickly fatal than disease acquired by the alimentary route. It is noteworthy that of the normal animals that survived the longest exposure and therefore had the greater natural resistance, those that finally acquired infection acquired it by way of the intestines. Furthermore it is interesting to find that alimentary infection was more common among the vaccinated animals whereas the respiratory infection was more common among the normal animals.

It is well known that as much as 1000 times the amount of bacilli is required to infect an animal through the alimentary tract as through the air passages. In 7 of the cages bacilli were simultaneously available for entry by both routes, and the quantity of bacilli that reached the intestines was greater than the quantity that penetrated to the alveoli, as is indicated by the following evidence. (1) The primary acquired lesion in the lung was usually single, as in man, but the primary lesion in the intestines was usually multiple. (2) The lymph nodes draining the acquired primary lesion in the lung were involved in only 1 of 8 vaccinated animals, whereas the mesenteric lymph nodes draining the primary lesion in the intestines of vaccinated rabbits were much more frequently involved. Nevertheless, owing to the greater vulnerability of the lung, a large percentage of normal rabbits early acquired a respiratory disease, which, as is well known, tends to inhibit the engrafting of the disease by the alimentary route. Normal rabbits of high natural resistance apparently destroyed the small numbers of bacilli that reached the lung only to succumb later to the *cumulative* larger dosage of bacilli that were absorbed in the lymph follicles of the appendix and ileocecal region. The same considerations explain the greater incidence of alimentary infection among the vaccinated rabbits.

These experiments emphasize anew the important rôle of native factors in resistance against tuberculosis, recently stressed by Lange (18). 27 per cent of normal rabbits completely escaped disease; 40 per cent of vaccinated animals escaped disease. The increased resistance afforded rabbits by vaccination with living virulent tubercle bacilli of the human type enhanced their natural resistance against undoubtedly large quantities of bacilli, but could save only an additional 13 per cent of them from fatal tuberculosis of exogenous origin, acquired by the natural mode of contagion. A study of the hereditary factors in natural resistance by the method described is now in progress.

SUMMARY

73 per cent of normal rabbits exposed for about 1 year to cage mates infected with tubercle bacilli of bovine type acquired a respiratory or alimentary tuberculosis, which was fatal in 50 per cent of the cases. 63.6 per cent developed tuberculosis during the first 6 months.

Of rabbits vaccinated with tubercle bacilli of human type and exposed in the same cages at the same time only 36.8 per cent acquired tuberculosis during the first 6 months. Later this resistance waned, and by the end of the year altogether 60 per cent had developed tuberculosis, of which 38 per cent succumbed.

The disease in the vaccinated rabbits was shown to be of exogenous origin by the isolation in pure culture from the same rabbit of the human type bacillus from the primary infection, and of the bovine type bacillus from the naturally acquired lesion.

The vaccination reduced the incidence, extent, and mortality of the disease, affected the route of infection, changed its pathological character, and retarded its progress. The disease acquired by vaccinated rabbits shared many characteristics with adult type tuberculosis in man.

It is suggested that this method may be used with relative ease in studying many phases of naturally acquired tuberculosis in small laboratory animals.

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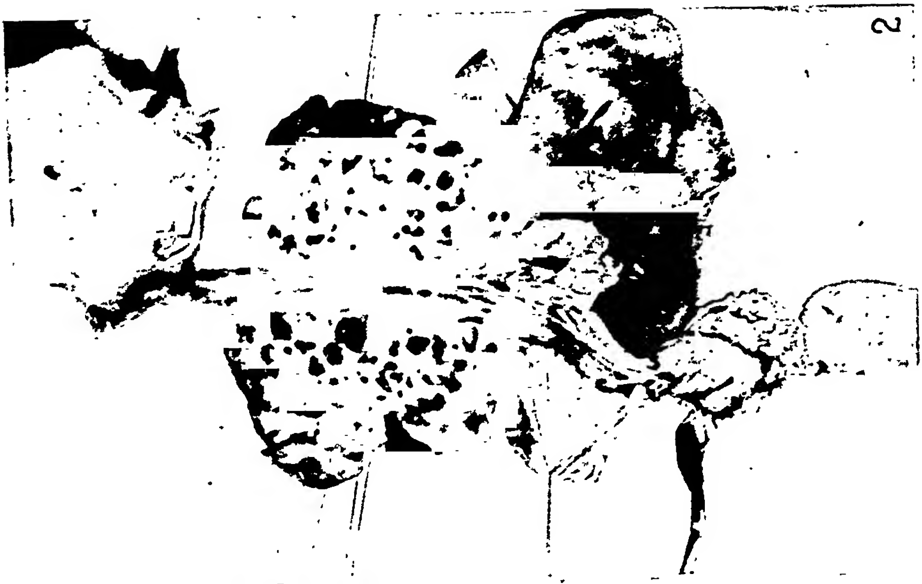
EXPLANATION OF PLATE 16

FIG. 1. The organs of normal Rabbit N-4-2, which died after 125 days of exposure in Cage 6 of massive caseous pneumonia. Note the extent of the disease in the kidney. The route of infection was both respiratory and alimentary.

FIG. 2. The organs of vaccinated Rabbit I-2, which died after 240 days of exposure in the same cage. The encapsulated subcutaneous lesion of vaccination with virulent human type tubercle bacilli is depicted in the upper right hand corner. The ulcer in the ileocecal junction is shown between the liver and kidney, in which there is a slight tuberculous lesion. Tuberculous nodules are shown in the mediastinum. The route of infection was enteric.



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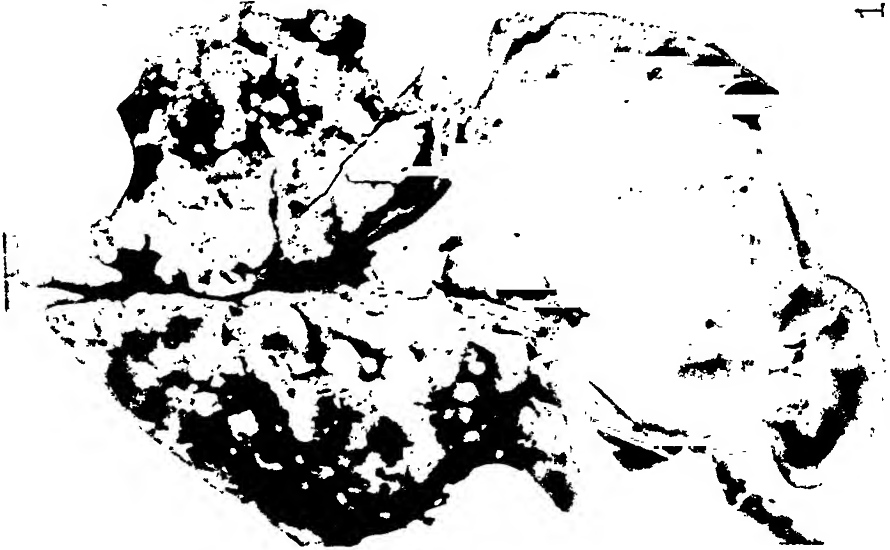

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(Lurie: Epidemiology of tuberculosis)

discussed by Löwenstein (8) in 1913. Dreyer (9) treated the organisms first with formalin, then extracted with acetone while heating, and repeated these procedures until the bacteria were no longer acid-fast. The diaplyte vaccine thus prepared was first thought to be quite efficacious and was employed clinically, as well as experimentally. Bronfenbrenner and Straub (10), and Douglas, Eddington, and Simson (11), however, found no beneficial effect from vaccination by Dreyer's method.

The vaccine used in the present experiments was prepared in the following manner.

Bovine tubercle bacilli were grown on Long's synthetic media for 3 weeks. To the bacilli, after they were harvested, equal parts of ether and 95 per cent alcohol were added. The mixture of ether, alcohol, and bacilli was saturated with CO_2 and allowed to stand with casual shaking for 4 months and 22 days. The alcohol-ether mixture was then decanted, the organisms were dried in the air, and ground for 48 hours in a ball mill. This fine powder was prepared by the Mulford Laboratories, Sharp and Dohme, Glenolden, Pennsylvania and was obtained through the courtesy of Dr. John Reichel. It contained a moderate number of intact bacilli, some of which retained their acid fastness. No viable tubercle bacilli could be demonstrated by cultures or inoculation of animals. This powder was ground thoroughly in a mortar with sterile 0.9 per cent saline, so that 1 cc. of the final suspension represented 0.2 mg. of the partial defatted organisms.

EXPERIMENTAL

Thirty rabbits were used in the experiment. Total and differential counts of the blood cells were made before any experimental procedure was begun. The differential counts were made by the supravital method. The weight of each animal was determined. After an adequate preliminary study of the blood had been made, ten of the animals were vaccinated with the partially defatted bacilli described above. Injections were done every 3rd day until each animal had seven injections. The volume of the suspension was in each instance 1 cc. At the time of the first injection each animal received 0.1 mg. of the partially defatted bacilli. The amount of each subsequent injection was 0.2 mg., so that each animal received a total of 1.3 mg. Half of the animals were vaccinated subcutaneously; the others intravenously. Blood studies were continued during and after the period of vaccination. 3 months and 8 days following the last injection of vaccine, the test and their control animals were inoculated intravenously with 0.1 mg. (moist weight) of a 16 day old subculture of bovine tubercle bacilli, Strain B-1. The animals were weighed and their blood cells were counted at irregular intervals from the time of inoculation until death. An autopsy was done on each animal and surveys were made of the macroscopic and microscopic pathology with special reference to the extent, distribution, and character of the lesions.

RESULTS

One vaccinated animal died 2 days following the last subcutaneous injection of the vaccine. From the autopsy, the fixed tissues, and from bacterial stains, it was found that the animal had a non-tuberculous pneumonia and empyema. In addition, one intravenously vaccinated animal was killed 15 days after the seventh injection for a study of the reaction to the defatted tubercle bacilli. The sections of lung from this animal showed numerous small masses of epithelioid cells surrounded by lymphocytes and a few giant cells, mostly of the foreign body type. The lesions were regressing, as many of the epithelioid cells showed evidence of degeneration. Tiny foci of caseation were present in some of the tubercles. A small number of rosette giant cells were seen also in the liver and spleen. The bone marrows were depleted and showed many abnormally young erythroid and myeloid cells. The latter observation was of interest in view of the changes in the blood cells due to the vaccinations.

Each of the animals vaccinated subcutaneously (in the groin) exhibited typical cold abscesses at the site of these vaccinations. In some instances these abscesses ruptured; in all instances the abscess or ulcer was healed at the time of inoculation with living tubercle bacilli.

Analyses of the blood counts of the vaccinated rabbits in the interval between vaccination and inoculation showed a most interesting sequence of events. All of the vaccinated animals developed an anemia and a leucopenia similar to that described by Sabin, Doan, and Cunningham (12), and by Sabin and Doan (13). The fall in total red and total white cells began during the vaccination period and continued 10 days after the last injection, after which there was a gradual return to values slightly higher than normal. The average total fall in red blood cells was about 1.4 millions (Chart 1); of the white cells 2,800 cells (Chart 2). Upon recovery the average white blood cell counts reached and were maintained at a level 2,500 cells higher than that existing before vaccination. From Charts 3 and 4 it will be seen that this overcompensation was due in part to higher values for granulocytes, but principally to a well marked lymphocytosis. Chart 4 also shows that the values for lymphocytes were maintained throughout the course of the disease at a level very significantly above that of the controls. The other types of white blood cells showed no significant difference from the controls during the postinoculation period. The partially defatted tubercle bacillus vaccine therefore contained the factors present in living bacilli which cause the anemia and leucopenia. In addition, there was the capacity to cause lymphocytosis that has been shown by Thomas to occur after the intravenous inoculation of rabbits with living tubercle bacilli (14, Fig. 6). In the present instance this lymphocytosis was sustained for a prolonged period.

The animals vaccinated with the partially defatted tubercle bacilli showed a very definite increase in resistance after inoculation with virulent living organisms. The average survival of the control animals was 167 days, while that for the vaccinated animals was 249 days—an increase of about 48 per cent. Moreover, the average survival time of the animals vaccinated intravenously was 319 days—an increase of 90 per cent above that for the controls. This was particularly significant in that the only intravenously inoculated animal which died under 100 days suffered a complicating non-tuberculous encephalitis (14). On Chart 5 is recorded the incidence of deaths in the vaccinated and control groups, by intervals of 15 days. This chart also shows the average survival of the two groups of animals.

The study of the lesions in the vaccinated and control animals showed that the former had somewhat fewer and less extensive lesions than the controls. Careful analysis with particular attention to the phase of the disease in which the animals died showed that this difference was not due to the diphasic nature of the disease (14). The greatest differences in extent and incidence of lesions were noted in the lymph nodes and bone marrow. In the vaccinated rabbits, only 10 of 51 lymph nodes were tuberculous (19.6 per cent). In the controls, 51 of 118 lymph nodes were tuberculous (42 per cent). In the vaccinated animals, 6 of 23 bone marrows (26 per cent) were tuberculous, whereas in the controls, 25 of 54 marrows exhibited the lesions (46 per cent). Furthermore, from Table I it will be seen that the vaccinated animals dying in the second 100 days of the disease showed no bone marrow or lymphadenoid tuberculosis, while lesions were common in the controls at this stage. Since bone marrow and

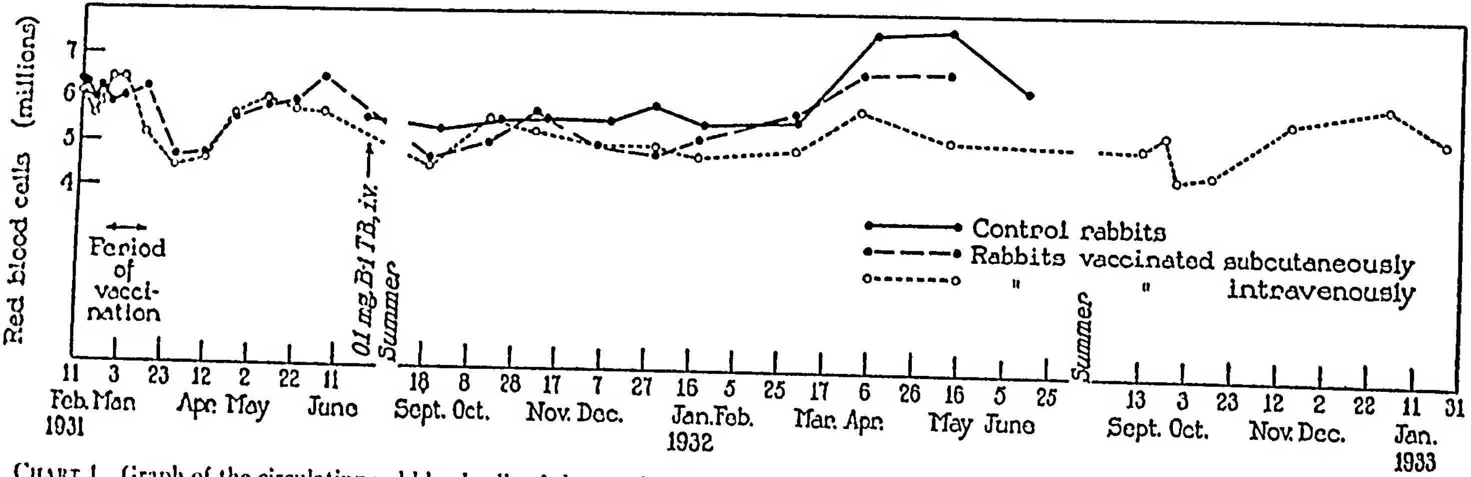

CHART 1. Graph of the circulating red blood cells of the vaccinated and control animals; the curves for both groups of vaccinated animals cover the periods before, during, and after vaccination as well as after inoculation with living tubercle bacilli, while that for the controls begins immediately before inoculation. The values on any day are expressed as averages for each group.

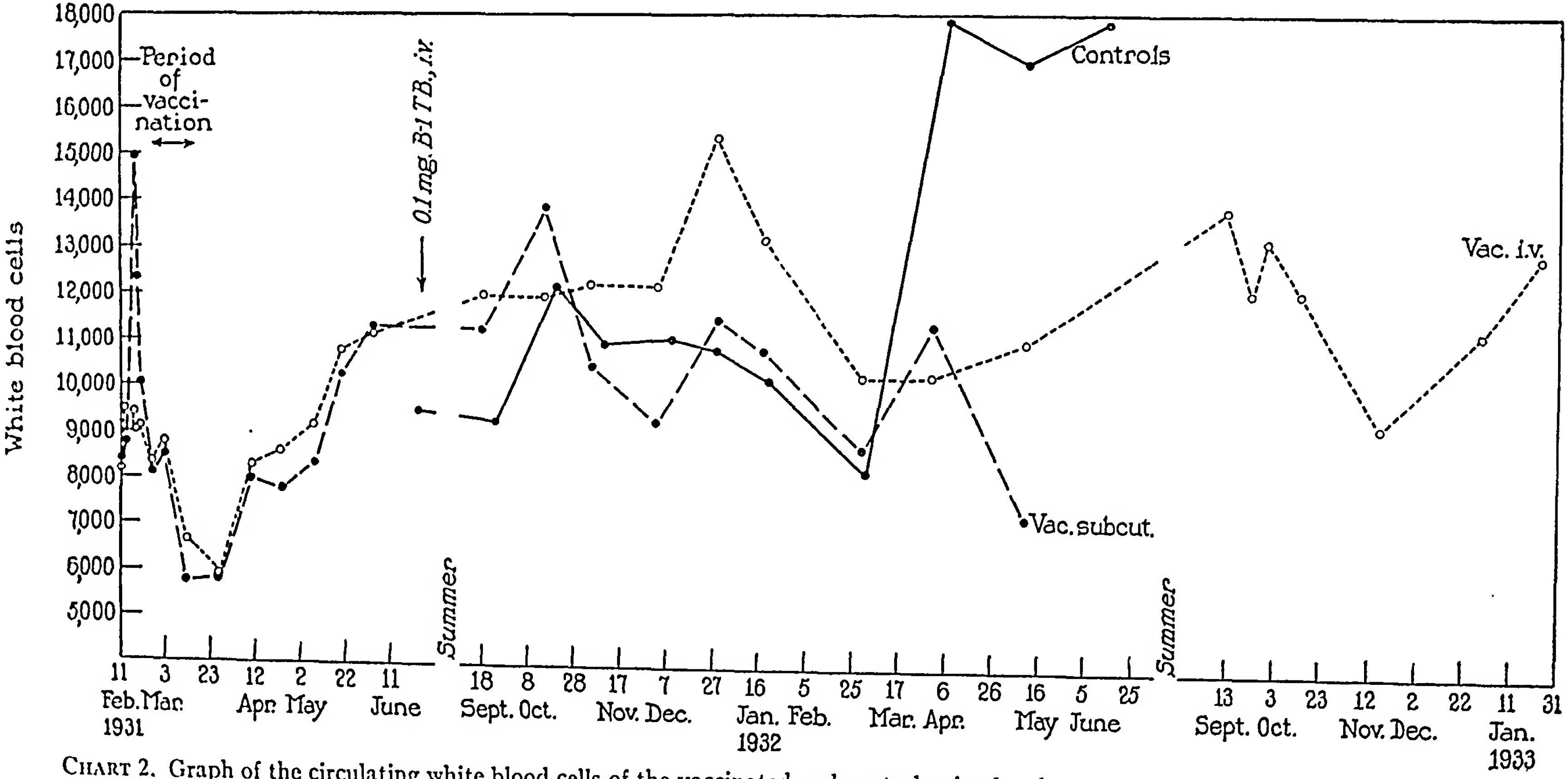

Chart 2. Graph of the circulating white blood cells of the vaccinated and control animals; the curves for both groups of vaccinated animals cover the periods before, during, and after vaccination as well as after inoculation with living tubercle bacilli, while that of the controls begins immediately before inoculation. The values are expressed as averages for each group. The sharp rise and fall in total leucocytes of the subcutaneously vaccinated animals were caused by an unexplained extreme leucocytosis in one of the group.

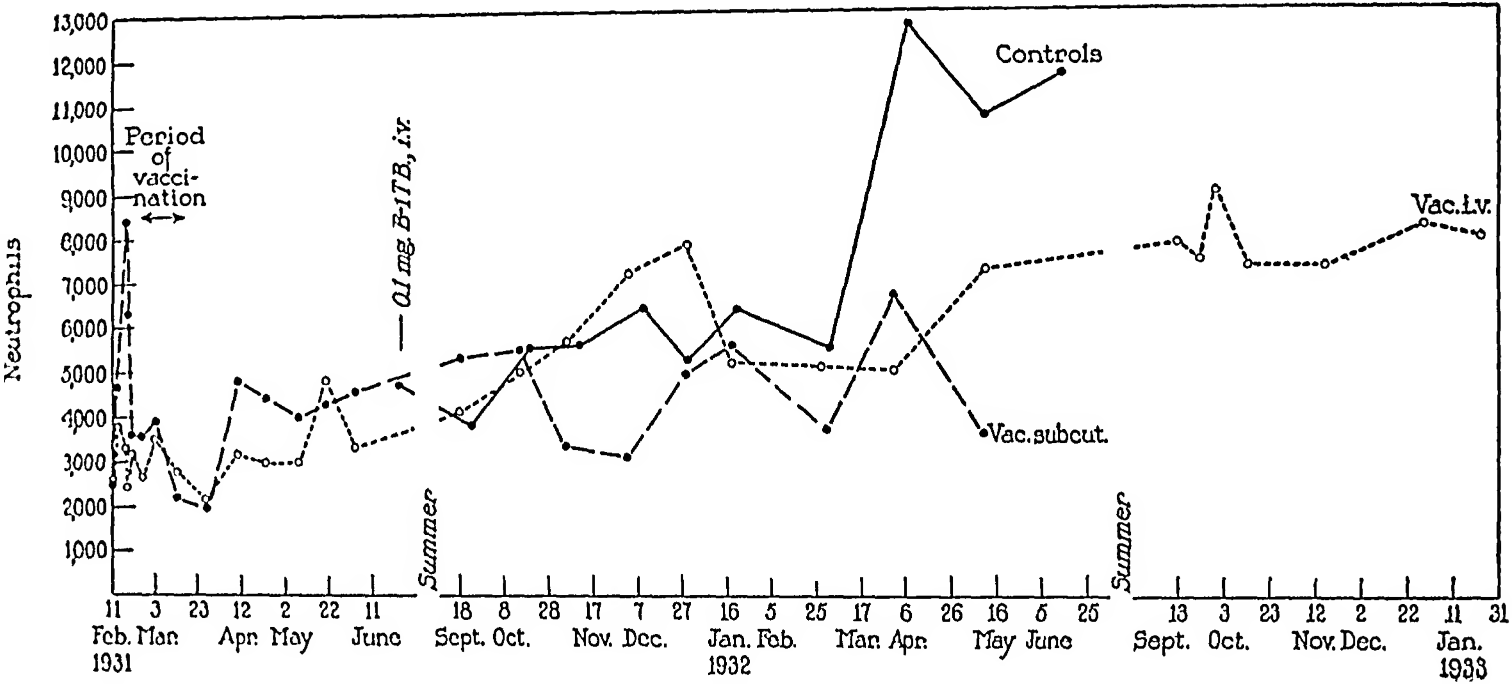

CHART 3. Graph of the circulating neutrophils of the vaccinated and control animals; the curves for both groups of vaccinated animals cover the periods before, during, and after vaccination as well as after inoculation with living tubercle bacilli, while that of the controls begins immediately before inoculation. The values shown are expressed as averages for each group.

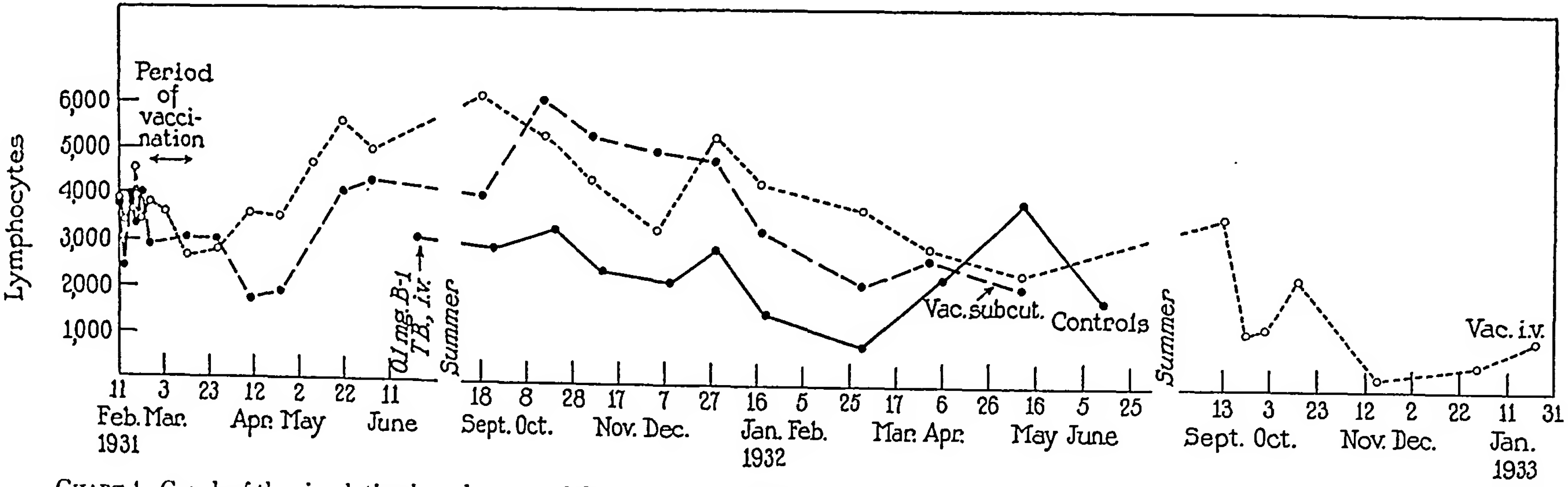

Chart 4. Graph of the circulating lymphocytes of the vaccinated and control animals; the curves for both groups of vaccinated animals cover the periods before, during, and after vaccination as well as after inoculation with living tubercle bacilli, while that for the controls begins immediately before inoculation. The values shown are expressed as averages for each group for any one day. Note the sustained high level of lymphocytes in the vaccinated animals as compared with their own normal values and those of the controls before and after inoculation.

lymph node lesions which occur after the 3rd month of the disease may be considered as metastatic (13), often arising from vascular lesions in remote parts of the body (15), it would seem that the power to localize the lesions was enhanced in these vaccinated animals. However, this power to localize the lesions was by no means absolute, as animals which died later in the disease showed numerous metastatic foci of tuberculosis.

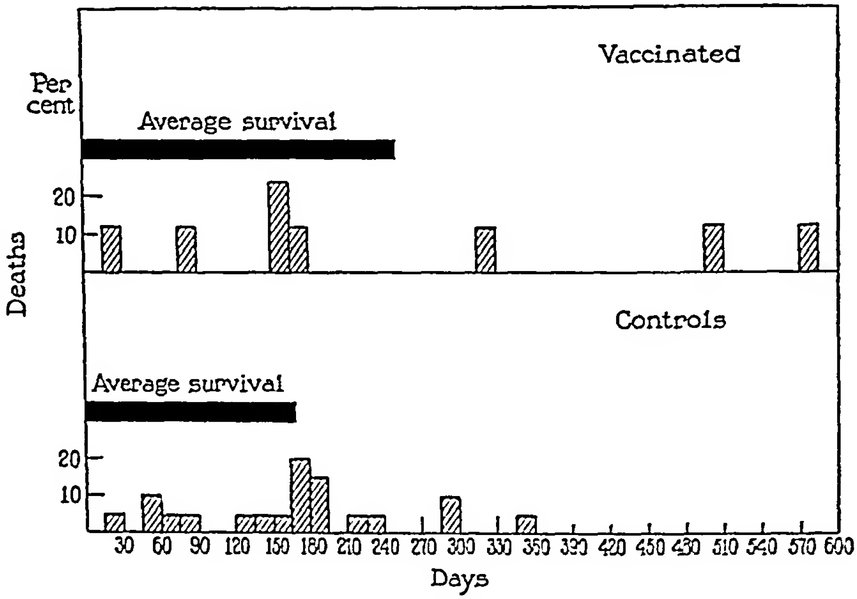

CHART 5. Incidence of deaths in the vaccinated and control groups in each 15 day period of the disease and the average survival time of the two groups in days.

It may be well to mention at this point that the vascular lesions referred to above, and previously described as occurring in the acute phase of the disease (15), were by no means limited to that phase. In the present experiments venous thrombi were seen in twelve animals—in one instance as late as 498 days after inoculation. These lesions were seen to occur either by direct extension of tubercles through the wall of a vein or by obstruction of lymphatic vessels by epithelioid cells and subsequent extension through the vein wall. Such vascular lesions were invariably present in those animals living longer than 3 months which showed tubercles in the bone marrows and peripheral lymph nodes.

Since Petroff (6) had shown that heat-killed tubercle bacilli render guinea pigs sensitive to tuberculin, it was decided to determine whether the partially defatted organisms possess this property. Accordingly, twelve non-tuberculin-reacting guinea pigs were divided into six pairs of two. One pair received a single intraperitoneal injection of 2.5 mg. of the defatted tubercle bacilli (human Strain H-37), one pair received two injections, and the other pair received three injections, each spaced by intervals of 5 days. The remaining six guinea pigs each received similar injections of heat-killed human tubercle bacilli. 28 days following the first injections, all the guinea pigs were tested with 0.1 mg. tuberculoprotein MA-100 intracutaneously. All tests were definitely positive. These were repeated 33 days later and were again positive, some more strongly than at the time of the first test. It is clear then that the defatted, as well as the heat-killed, tubercle bacilli can induce cutaneous hypersensitiveness to tuberculoprotein. Moreover, a single injection of 2.5 mg. of either is sufficient to sensitize.

TABLE I

Incidence of Tuberculous Lesions in Lymph Nodes and Bone Marrows of the Vaccinated and Control Animals Dying at Various Stages of the Disease

	0–100 days		100–200 days		200+ days	
	Vaccinated	Controls	Vaccinated	Controls	Vaccinated	Controls
Lymph nodes						
No. sections	8	23	20	60	23	35
No. tuberculous	4	13	0	30	6	8
Per cent tuberculous	50	56.5	0	50	26	23
Bone marrow						
No. sections	5	11	9	28	9	15
No. tuberculous	1	5	0	16	5	4
Per cent tuberculous	20	45	0	57	55	27

The prophylactic value of the defatted tubercle bacillus vaccine is being studied more extensively at the present time.

SUMMARY

1. A vaccine prepared from partially defatted bovine tubercle bacilli influenced favorably the survival time after inoculation with living virulent bovine tubercle bacilli.

2. Accompanying this increased resistance was a sustained lymphocytosis in the vaccinated animals.

3. The vaccine induced a transitory anemia and leucopenia.

4. A similar preparation of partially defatted human tubercle bacilli possessed the power to sensitize guinea pigs to tuberculo-protein.

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STUDIES ON MENINGOCOCCUS INFECTION

III. The Antigenic Complex of the Meningococcus—a Type-Specific Substance

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In the two preceding papers, it has been shown that freshly isolated strains of meningococcus differ not inconsiderably from those strains maintained in the laboratory on artificial media over long periods, and known as stock strains. Amongst the observations which have been recorded is the ability of fresh strains to evoke the production of precipitin-containing sera (1) which will react specifically with the type-specific substances occurring in these same fresh strains (2). This and other related observations pointing to the probability that the meningococcus has an antigenic complex comparable, in its essentials, to that described for other organisms—especially those of the non-encapsulated group (3)—are described in some detail in this and the following paper which deal with the isolation and analysis of some of the antigenic components.

That precipitation reactions between soluble extracts of the meningococcus and antimeningococcal sera occur and are of some importance diagnostically is no new observation. As long ago as 1906, Bruckner and Cristeanu (4) showed that, while extracts in 0.75 per cent NaCl are but feeble precipitinogens, extracts of meningococci prepared in 0.15 per cent NaOH give good precipitates on the addition of antimeningococcal serum, but not with normal horse serum. They showed, moreover, that the meningococcal extract precipitates with antigonococcal serum and that the same cross-precipitation occurs when an alkaline extract of gonococci is set up with antimeningococcal and antigonococcal sera. Dopter and Koch (5) confirmed these results and introduced the conception of co-precipitins to account for the cross-reactions. They showed that when the antimeningococcal serum is absorbed with whole meningococci both the specific meningococcus precipitins and also the group precipitins, which react with the gonococcus extract, are removed; on the other hand, when the serum is absorbed with whole gonococci, only the group precipitins are removed and the absorbed serum will still react with the meningococcus extract. In the same paper they showed that the reverse is true and that whole gonococci remove both specific and group precipitins from antigonococcal serum, while whole meningococci remove only the group precipitins. In a subsequent paper (6), Dopter extended these observations. He stated that precipitating sera can be obtained by means of intravenous injection of either living organisms or meningococcus extract, and that the former are often the more efficacious. The titre obtained varies with the animal immunized and the strain used for vaccination, and is not always highest when a readily agglutinable strain is used. The precipitin usually parallels the agglutinin titre, but this is not invariably the case. He showed that cross-precipitation of antimeningococcal serum, often in high dilution, occurs with all members of the Gram-negative pharyngeal micrococcus group but that, as is the case with the gonococcus, the cross-reaction is due to the presence of group precipitins which can be removed by absorption with any member of the group, while the meningococcus alone can remove the specific antibodies from the serum. He pointed out that precipitation occurs even at room temperature. Still later, Dopter (7) showed that, in general, the extract of each type (A, B, C and D) reacts only with its homologous antiserum. He found, however, not infrequent exceptions in which cross-precipitation occurred, though the titre in such cases is higher in homologous than in heterologous sera. Absorption of anti-A serum with either Type A organisms or extract removed the homologous precipitins and the same is true for the absorption of other type sera by their homologous strains.

Zinsser and Parker (8) obtained a residue antigen from the meningococcus by alkaline extraction of young and old broth cultures. Przesmycki, in a brief communication (9), showed that specific residue antigens can be obtained for each of the four types by precipitation with five volumes of absolute alcohol. He found that these specific antigens react most strongly with their homologous antisera, but do show cross-precipitation with heterologous sera at lower titre—results which correspond closely with those of Dopter. Przesmycki made but few tests and himself stated that no definite conclusions could be drawn from his results. Recently Zozaya (10) has studied polysaccharide and protein fractions obtained from the different types of meningococci and compared them with fractions obtained from pneumococci and Gram-negative cocci. The polysaccharide is obtained from alkaline extracts of the organisms by precipitation with four to five volumes of ethyl alcohol, while the nucleoprotein is isolated by 10 per cent acetic acid precipitation of a watery solution of organisms which have been frozen and thawed. He found cross-precipitation of the polysaccharides of meningococcus, gonococcus and *Micrococcus catarrhalis* with polyvalent antimeningococcal serum and showed that these group precipitins could be removed by absorption of the serum with any one of the three polysaccharides. There was no precipitation of the polysaccharides of pneumococcus and of Bargen's organism with antimeningococcal serum, nor did the meningococcus polysaccharide react with antipneumococcal and anti-Bargen sera. Polysaccharides from each of the meningococcus types showed equal precipitation with polyvalent antimeningococcal serum, and furthermore, absorption with Type I polysaccharide removed polysaccharide precipitins for all types. This polysaccharide is, therefore, in no way type-specific. The nucleoprotein was also found to be common to the group rather than type-specific. It does not precipitate with sera of organisms outside the group of Gram-negative cocci and absorption with nucleoprotein of Type I removes the nucleoprotein precipitins for all the types. In a further paper (11), Zozaya found similarities in the polysaccharides of the meningococcus and certain Gram-positive bacilli (anthrax, *proteus*, *mesentericus* and *subtilus*). Absorption of polyvalent antimeningococcal serum with the polysaccharide of any one of these organisms removes the precipitins for the meningococcus polysaccharide. On the other hand, only absorption with anthrax polysaccharide will remove the anthrax precipitins from anti-anthrax serum, while the other polysaccharides are unable to do this. The presence of at least two carbohydrate antibodies is presumed, one species-specific and the other group- or non-specific. It is of interest to note that in no case was the agglutination titre of the absorbed serum altered from that of the unabsorbed control and this even when the absorption of precipitin was successful.

As this brief review of the previous studies on precipitable substances of the meningococcus indicates, the findings may be divided conveniently into two periods. In the first, no attention was paid to the chemical complex of the various extracts. The earlier investigators showed that alkaline extracts of the meningococcus contain precipitinogens which react with both antimeningococcus serum and the serum prepared against others of the Gram-negative cocci (4–6); that the antisera contain both species-specific precipitins which can be removed only by the homologous organism or its extract, and group precipitins which can be removed by any of the organisms or their extracts (5, 6), and finally, that there is evidence of the presence of type-specific precipitinogens as contrasted with those which are only species-specific and common to all the meningococcus types (7). In the later period, commencing with the work of Zinsser and Parker (8), endeavors have been made to isolate and identify the different fractions of the meningococcus extract which are responsible for the varying reactions observed. It has been shown that precipitation with 10 per cent acetic acid will give a nucleoprotein fraction which is species-specific (2, 10), while precipitation with ethyl alcohol gives at least two fractions of which one is type-specific (2, 9) while the other is related to similar substances present in and obtained from other microorganisms, especially those of the Gram-negative coccus group (2, 10, 11).

Material and Technique

Several methods of extracting the meningococci were tried. These included extraction with normal saline and also with N/20 HCl. The most satisfactory, as far as yield of the various fractions and comparative ease of separation are concerned, was that of autolysis.

The organisms are planted in 9 cc. of beef heart infusion broth at pH 7.6. At the end of 24 hours, or as soon as good growth is apparent, the whole 9 cc. are planted in a liter flask containing from 700 to 900 cc. of hormone broth. If growth takes place, a pellicle becomes obvious on the surface of the broth in 2 or 3 days, which breaks up into fine granules and gradually settles to the bottom of the medium when the flask is disturbed. A new pellicle now forms on the surface and by daily shaking of the flask a constant new growth may be obtained at the surface, while those organisms which have settled out undergo gradual autolysis at the bottom. Growth is maintained at 37°C. for about 2 weeks.

The broth culture is now centrifuged at high speed to remove all debris. The supernatant, slightly cloudy fluid is measured in a graduated cylinder and then placed in a liter flask. 10 cc. are taken in a test-tube, and to this is added at room temperature from a burette 10 per cent acetic acid drop by drop until no more precipitation occurs. A simple reckoning will permit the adding of sufficient 10 per cent acetic acid to the bulk of the autolysate to throw out of solution the acetic acid-precipitable fraction. This appears as a greyish white deposit. The mixture is centrifuged, the clear supernatant fluid decanted and the precipitate, labelled P, is preserved.

A strong solution of sodium hydroxide is added to the supernatant fluid until it is neutral to litmus. It is now treated with 95 per cent ethyl alcohol. When an equal volume of alcohol is added, a slight precipitate appears and this becomes heavy when the amount of alcohol reaches two to two and a half volumes. This amount of alcohol is allowed to stand in contact with the broth solution for 24 hours at room temperature. During this time a white flocculent precipitate settles out completely. It is packed by centrifugation at high speed and the supernatant fluid decanted. This fraction, for reasons to be shown later, is termed the specific substance. It is dried *in vacuo* over phosphorus pentoxide, when it forms a brownish white material somewhat like gelatin in appearance. Most of the first precipitate is insoluble in distilled water or saline, but all or apparently all of the active material goes into solution after standing in contact with distilled water or saline for a day or two. The clear, somewhat brownish solution is reprecipitated with two and a half volumes of 95 per cent ethyl alcohol. The yield of this precipitate varies between about 250 and 500 mg. of dried material from 900 cc. of broth. It is dried *in vacuo* as before and redissolved in saline or distilled water. On this occasion about 90 per cent of the precipitate goes into solution; *i.e.*, in one case 390 mg. out of 436 mg. and in another 209 mg. out of 224 mg. The substance may be further purified by repeated alcohol precipitation. A few crystals of sodium acetate should be added to every alternate solution-alcohol mixture in order to facilitate, indeed to ensure, complete precipitation. A standard solution containing 10 mg. of specific substance in 1 cc. of solution, *i.e.* 1/100, is made up, a few drops of toluol are added and it is stored in the ice box to be used as required.

To the supernatant fluid decanted from the specific substance precipitate are added another six volumes of 95 per cent ethyl alcohol. A heavy white precipitate settles out and is allowed to stand in contact with the supernatant alcohol mixture for at least 24 hours at room temperature. It is now centrifuged and the supernatant fluid is decanted. The precipitate is dried over phosphorus pentoxide *in vacuo* and forms a white, caked powder. Most of this redissolves readily in water. It is further purified by repeated alcohol precipitation followed by resolution, sodium acetate being added as above. After the first two occasions all but the merest trace goes back into solution. This fraction is labelled C.

The Type-Specific Substances

The fraction precipitable by means of two to two and a half volumes of ethyl alcohol has been obtained from Type I, Type II and Type III strains. Difficulty was experienced in obtaining a strain of Type III organisms. At the present time, the great majority of strains which occur are either Type II or belong to what may be termed the I-III group. The members of this latter group agglutinate equally well with both Type I and Type III monovalent antisera and, as will be demonstrated below, show even closer relationships when examined by serum absorption experiments. There is general agreement among those working with this organism that, whatever may have been the case some years ago, at the moment great difficulty exists in separating out two types from the I-III group, and it has been conceded that the strains which occur at the present time correspond most closely to what was formerly termed Type I. Some laboratories continue to report Type III strains, but the writer has not found such a strain among the freshly isolated spinal fluid and throat strains which have been received. Through the kindness of Dr. Sara Branham of Washington, a Type III strain (No. 302), isolated in 1930, was obtained. This is said to be the most narrowly specific Type III strain at present in the United States Public Health Service Department. It has been used for the preparation of the Type III specific substance and is fully discussed below. No freshly isolated strain of Type IV meningococcus has been available up to the present time and, apparently, few, if any cases of meningitis due to this organism are occurring at the moment.

Type I

The type-specific substance has been obtained from several Type I strains. Most of the work, however, has been carried out on the fraction obtained from a hormone-broth autolysate of Strain 428 which had grown in 900 cc. of the broth for 2 weeks. The method of preparation has been described above.

This Strain 428 showed the characteristic agglutination of a freshly isolated Type I strain.[1]

The agglutination test is carried out for 2 hours at 37°C. and overnight in the ice box. The readings given are the final ones. All the monovalent sera are prepared from freshly isolated spinal fluid strains (1) with the exception of the Type IV serum which, in the absence of any fresh strains of this type, is prepared from a stock strain isolated in March, 1929.

TABLE I

Dilutions	Serum			
	I	II	III	IV
	No. 578	No. 551	No. 556	No. 535
1/10	+++	0	+++	0
1/25	+++	0	++	0
1/50	+++	0	++	0
1/75	++	0	+	0
1/100	++	0	+	0

Saline—negative

The fraction gives a good precipitin reaction with homologous antisera. Little or no difference can be found between the reaction with Type I and that with Type III sera, either in the amount or the type

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[1] All precipitin tests have been carried out in the same way. Equal parts—0.1 cc.—of serum and solution of the fraction to be investigated have been placed with a capillary pipette in a small precipitin tube 4 to 7 mm. in diameter. The serum is placed at the bottom of the tube and the solution carefully layered on top to form a clear line of junction. The reading is made immediately and then after the tube has stood for 1 hour in the 37°C. water bath. This constitutes the ring test. The two fluids are then mixed and returned to the bath for 1 hour, when they are read again and then placed in the ice box overnight for a final reading.

of precipitate formed. On the other hand, there is little cross-precipitation with Type II and Type IV antisera, and whatever cross-precipitation exists can be removed by appropriate absorption of the sera.

Experiment 1.—A purified specimen of Type I specific substance is made up in a dilution of 1 in 200. This is then set up against specimens of the four monovalent antisera.[1] In the first case, the antisera have not been treated. In the second case, 0.5 cc. of each of the monovalent sera has been absorbed (1) with 0.15 cc. of packed Type II organisms, and (2) with 5 mg. of dried C substance (prepared from Strain 23, Type I meningococcus). These absorptions were carried out on separate occasions. The organisms stood in contact with the serum for 18 hours at 37°C., the C substance for 12 hours at 37°C. and 12 hours in the ice box.[2] The results of the precipitin experiments with both unabsorbed and absorbed sera are shown in Table II.

It will be seen that the fraction obtained from Type I organisms gives a good precipitate with homologous unabsorbed Type I or Type III sera and also a slight precipitate with unabsorbed Type II or Type IV sera. Absorption of the sera in the manner indicated produces a much clearer result in that it removes all the precipitins in the heterologous sera but leaves those in the homologous I and III sera unaffected. In this particular experiment the Type I serum used had a rather low precipitin titre which accounts for the difference in the amount of precipitate formed with this serum as compared to that of Type III.

It will also be noted that the type of precipitate formed with the homologous sera is different after absorption, being now in the form of a partial disc whereas formerly it was finely granular. The exact significance of this cannot be determined at present, but it can be said that the purer the specimen of specific substance the more liability there is for the formation of a partial disc or even a true disc in the

[2] As has been the experience of other workers with other sera, it has been found difficult to absorb any antimeningococcal sera completely, no matter what the method employed or the number of times it is repeated. Theoretically, it should be sufficient in this particular case to absorb the sera with the heterologous Type II whole organisms. In practice, however, even repeated absorption with these organisms fails to remove all the non-specific precipitins in the sera and the additional absorption with C substance has been found greatly to improve the results.

precipitin reaction. It would seem that the presence of impurities being precipitated with the type-specific substance changes the character of the precipitate and renders it granular.

It might be supposed that the cross-reactions which occur between the type-specific substance and heterologous unabsorbed antisera (not including under this term the Type III antiserum) are due to the presence in the purified fraction of small amounts of non-type-specific carbohydrate C and nucleoprotein P fractions, and the presence in the monovalent antisera of their corresponding antibodies. This is no doubt the case to some degree, yet experiments such as Experiment 2 have shown that the monovalent antisera contain such insignificant amounts of the anti-C and anti-P antibodies that one must doubt whether these are altogether responsible for the cross-reactions.

TABLE II

Sera unabsorbed				Sera absorbed					
I	II	III	IV	I	II	III	IV		
No. 578	No. 551	No. 266	No. 535	No. 578	No. 551	No. 266	No. 535		
$+^{r}$	$\pm^{r}$	$+^{r}$	$\mp^{r}$	$+^{r}$	0	$+^{r}$	0	Immediate	Ring test
$+\pm^{p}$	$+^{r}$	$+++^{p}$	$\pm^{r}$	$^{p}++^{r}$	0	$^{p}+++^{r}$	0	1 hr.	
$+\pm^{p}$	+	$+++^{p}$	±	$++^{p}$	0	$+++^{pd}$	0	2 hrs.	
$++^{p}$	$+\pm^{p}$	$++++^{p}$	±	$++^{pd}$	0	$++++^{pd}$	∓	Ice box	

r = ring.
p = granular precipitate.
pd = disc broken up on agitation.
∓
±
+
+± = increasing amounts of precipitate.
++
+++
++++

Experiment 2.—The nucleoprotein fraction (of Strain 31, Type II, purified by three precipitations with 10 per cent acetic acid) was made up in a standard 1/100 solution. A specimen of C substance (from Strain 7, Type I-III, purified by three precipitations with 95 per cent ethyl alcohol) was also prepared in a dilution of

1/100. These two were set up with the four monovalent sera and with a specimen of polyvalent serum. The results are shown in Table III.

It will be seen from the table that although these group-specific fractions react strongly with the polyvalent serum they give only very slight reactions with the monovalent sera. With the P fraction indeed the reaction is only with the homologous serum and this, as will be shown later, is due to impurities of the type-specific substance, great difficulty being experienced in freeing the P fraction altogether from this component. These facts suggest that the cross-precipitation seen with some specimens of type-specific substances may be due to some antigen other than those as yet isolated. The antibodies for this antigen can be absorbed by using whole organisms, as was shown in Experiment 1.

TABLE III

P fraction $31P^3$ (Type II)					C fraction $7C^3$ (Type I-III)					
I	II	III	IV	Polyvalent	I	II	III	IV	Polyvalent	
No. 578	No. 551	No. 556	No. 535	No. 2	No. 578	No. 551	No. 556	No. 535	No. 2	
0	$\pm^{cr}$	0	0	$\pm^{cr}$	$\pm^{r}$	$\pm^{cr}$	0	$\mp^{r}$	$++^{r}$	Immediate } Ring test
0	$\pm^{cr}$	0	0	$\pm^{cr}$	$\pm^{r}$	$\pm^{r}$	0	$\pm^{r}$	$^{d}++++^{r}$	1 hr.
0	$\pm$	0	0	$\pm$	$\pm$	$\pm$	$\mp$	$\pm$	$+++^{d}$	2 hrs.
0	$\pm$	0	0	$++++^{p}$	$\pm$	$\pm$	$\mp$	$\pm$	$++++^{d}$	Ice box

c = cloudiness.
r = ring.
p = granular precipitate.
d = firm disc.

The specificity of the precipitin reaction between the type-specific substance and the monovalent sera can be brought out by a method other than that of absorption; namely, by dilution.

Dilutions may be made either of the solution of type-specific substance with the serum remaining constant, or of the serum with the dilution of type-specific substance remaining constant. Under both of these circumstances the titre is higher with the homologous sera (including Type III serum) and the amount of precipitate formed is greater with such sera.

Experiment 3.—Dilutions of the type-specific substance of Type I (Strain 428), which had been purified by reprecipitation, were made from 1/100 to 1/10,000. These were set up against undiluted serum. Serum dilutions were made from undiluted serum to 1 part in 32. All dilutions were made in normal saline. The serum dilutions were set up against a 1/1,000 solution of type-specific substance. The results are shown in Table IV.

It will be noted that the specific precipitation occurs with dilutions of specific substance of 1/10,000 and undiluted serum, or with dilutions of serum of 1/32 and specific substance 1/1,000. On the other hand, the heterologous reaction occurs only at 1/1,000 of specific substance dilutions and 1/8 of serum dilution. Moreover, the amount of precipitate formed in the cross-precipitation is throughout more scanty. It will further be seen that the Type III antiserum gives a higher titre in both parts of the experiment than the Type I serum. The specimen of Type III antiserum used in this experiment had the highest titre of any rabbit monovalent serum prepared up to the time the experiment was carried out.[3]

The Type I specific substance precipitates readily with some specimens of polyvalent antimeningococcal horse serum. The titre reached is often higher than that obtained with the homologous monovalent rabbit serum.

Experiment 4.—Dilutions of the type-specific substance from 1/100 to 1/10-000,000 were set up against undiluted polyvalent antimeningococcal horse serum No. 3. Dilutions of the serum from 1/1 to 1/32 were set up against type-specific substance diluted 1/1,000. The precipitin test was carried out in the usual way. Table IV shows the final readings.

It will be seen from Table IV that the type-specific substance precipitates well with the particular sample of polyvalent antimeningococcal horse serum used in this experiment. With dilutions of the specific substance itself, a higher titre is reached with the polyvalent serum than with the homologous antisera, while in the serum dilutions the titre is the same in both instances. From this it might appear that polyvalent sera contain more type-specific precipitins than do the specifically prepared monovalent sera. Absorption experiments, how-

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[3] Specimens of Type I specific substance prepared more recently by improved chemical methods give a good precipitate in homologous sera at 1/1,000,000 and fail to precipitate with Type II or Type IV sera at 1/1,000.

TABLE IV

Serum		Dilutions of type-specific substance						Serum		Dilutions of serum					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}			1/1	1/2	1/4	1/8	1/16	1/32
Type I serum	Serum 1/1	+++^{p}	+++^{p}	+^{p}	0	0	0	Type I serum	Type-specific substance 10^{-3}	+++^{pd}	++^{p}	±	±	0	0
Type II serum		++^{p}	+^{p}	0	0	0	0	Type II serum		±	∓	∓	∓	0	0
Type III serum		++++^{pd}	++++^{pd}	++^{p}	0	0	0	Type III serum		+++^{pd}	+++^{pd}	++^{p}	+^{p}	±	∓
Type IV serum		+^{p}	±	0	0	0	0	Type IV serum		±	±	∓	∓	0	0
Polyvalent serum		++++^{pd}	++^{p}	++^{p}	±	0	0	Polyvalent serum		++^{p}	++^{p}	++^{p}	+^{p}	∓	∓

Only the ice box readings are given.

p = granular precipitate.

ever, prove that such is not the case, for heterologous strains or specimens of non-type-specific fractions will remove a larger part of these precipitins. They are, therefore, not type-specific and the precipitates formed represent in large degree reactions with impurities remaining in the sample of type-specific substance.

Two samples of polyvalent serum No. 3 were absorbed twice with large amounts of homologous (Type I, Strain 454) and heterologous (Type II, Strain 442) whole organisms for 2½ hours at 37°C. and overnight in the ice box. The polyvalent absorbed sera were set up against dilutions of the type-specific substance. Not even the homologous absorption removed all the precipitins from the serum. The heterologous strain removed a large part of the precipitins, showing that they were group-specific rather than type-specific; yet the absorption with homologous organisms was even more complete, showing that a part of the precipitins in the polyvalent antimeningococcal horse serum are, as might be expected, type-specific.

The precipitin reaction between the type-specific substance and the monovalent rabbit antiserum shows an inhibition zone in the lower dilutions. This phenomenon is brought out very clearly by the method of precipitin test that has been used—namely, first a ring test and then mixing of the sera. No zone phenomenon is shown by the ring test, but when lower dilutions of the type-specific substance have been used, reabsorption of the precipitate occurs in quite a dramatic manner directly the solution of specific substance and serum are mixed intimately.

Experiment 5.—Dilutions of Type I specific substance were made from 1/40 to 1/125. These were set up against undiluted Type I monovalent serum and the precipitin test was carried out in the usual way.

At the end of 1 hour at 37°C., there is no inhibition zone demonstrated by the ring precipitin test. Directly on mixing, however, in the three lower dilutions all precipitate formed disappears completely, the precipitate in the fourth tube is decreased slightly, while that in the fifth tube, *i.e.*, the highest dilution, remains unchanged. At the end of the 2nd hour at 37°C., the first three tubes show very little precipitate. The precipitate in the fourth and fifth tubes is about equal. After standing overnight in the ice box the amounts of precipitate in all tubes are about equal.

The type-specific substance in a dilution of 1/100 gives a slight precipitate with both antigonococcal and antipneumococcal Type III sera. There is no reaction at 1/1,000 or higher dilutions. The reaction is without doubt due to the incomplete purification of the fraction.

Chemical Analysis.—A more detailed chemical study of the various fractions thus far isolated is being undertaken at present and the results will be presented in a later paper (12). Crude specimens of Type I specific substance give a positive biuret test, a precipitate with 20 per cent trichloracetic acid and a Molisch reaction. The most purified specimen yet obtained, giving a precipitin reaction out to 1/10,000 with homologous rabbit serum and 1/100,000 with polyvalent horse serum, showed the following on analysis. A Molisch reaction could be obtained in a dilution of 1/100; and a strong biuret test and a marked precipitate with trichloracetic acid were obtained in a dilution of 1/1,000, though both tests were negative at 1/10,000.

TABLE V

Dilutions	Serum			
	I	II	III	IV
	No. 578	No. 551	No. 556	No. 535
1/10	0	+++	0	0
1/25	0	+++	0	0
1/50	0	++	0	0
1/75	0	++	0	0
1/100	0	++	0	0
Saline—negative				

Type II

In this case also, most of the work has been carried out with a single specimen of type-specific substance obtained from Strain 31 in the manner already described. This strain showed a characteristic agglutination (Table V) in monovalent sera prepared with freshly isolated strains (1).

The fraction gives a good precipitin reaction with homologous Type II monovalent rabbit antiserum with rather less cross-precipitation than is obtained with the specimen of type-specific substance obtained from Type I. A noticeable feature is a quite marked cross-precipitation with Type IV antiserum—more marked than is the case with Type I specific substance. This suggestion of relationship of Type II to Type IV has been noted several times throughout the investigations; owing, however, to the absence at the present time of a freshly isolated Type IV strain, the serum used is not of the same value as that for Types I, II and III, nor can the problem be investigated further at the moment. Absorption of the monovalent sera removes all the group precipitins and, it must be noted, removes the precipitins of the Type IV serum.

TABLE VI

Sera unabsorbed				Sera absorbed					
I	II	III	IV	I	II	III	IV		
No. 578	No. 551	No. 556	No. 535	No. 578	No. 551	No. 556	No. 535		
0	$+^{or}$	0	0	0	$+^{cr}$	0	0	Immediate	Ring test
$+^{r}$	$^{p}+++^{r}$	$+^{r}$	$+\pm^{r}$	0	$++^{cr}$	0	0	1 hr.	
+	$+++^{p}$	+	$++^{p}$	0	++	0	0	2 hrs.	
+	$++++^{pd}$	+	$++^{pd}$	0	$+++^{p}$	0	0	Ice box	

Experiment 6.—A purified specimen of Type II specific substance is made up in a dilution of 1 part in 100 of saline. This is set up against unabsorbed and absorbed monovalent rabbit sera and the precipitin test carried out in the usual manner. As in Experiment 1, the absorption was carried out both with dried C substance and with whole organisms, on this occasion of Type I (Table VI).

As with Type I specific substance, dilution of either the solution of specific substance or of the serum serves to bring out the specificity of the reaction.

Experiment 7.—Dilutions of a purified specimen of Type II specific substance were made, ranging from 1/100 to 1/10,000,000. These were set up against undiluted serum. Serum dilutions were made from undiluted serum to 1 part in 32 and these were set up against a 1/1,000 solution of the specific substance (Table VII).

It will be noted that the type-specific substance reacts with the homologous serum in a dilution of 1/100,000 albeit slightly. This is

TABLE VII

Serum		Dilutions of type-specific substance						Serum		Dilutions of serum					
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}			1/1	1/2	1/4	1/8	1/16	1/32
Type I serum	Serum 1/1	+±	+	∓	0	0	0	Type I serum	Type-specific substance 10^{-3}	$+^{p}$	$+^{p}$	±	∓	0	0
Type II serum		$++++^{pd}$	$+++^{p}$	±	∓	0	0	Type II serum		$+++^{p}$	$++^{p}$	$++^{p}$	+	±	∓
Type III serum		$++^{p}$	$+^{p}$	0	0	0	0	Type III serum		$+^{p}$	$++^{p}$	$+^{p}$	±	∓	0
Type IV serum		$++^{p}$	$+\pm^{p}$	∓	0	0	0	Type IV serum		$++^{p}$	$++^{p}$	$+^{p}$	+	±	0
Polyvalent serum		$+++^{pd}$	$++^{p}$	∓	0	0	0	Polyvalent serum		$++^{p}$	$++^{p}$	$++^{p}$	$+^{p}$	±	±

Final readings shown.

to be compared with the high dilutions in which this same specific substance gives protein reactions (see below). In the serum dilution protocol, the relationship of the Type IV to the Type II already mentioned above is brought out quite sharply.

As with the Type I specific substance, so the Type II reacts with some specimens of polyvalent antimeningococcal horse serum. This reaction is due in part to type-specific and in part to group-specific precipitins in the serum, as can be shown by absorption.

Experiment 8.—Dilutions of Type II specific substance from 1/100 to 1/10-000,000 were set up against undiluted polyvalent serum No. 3. Dilutions of serum from undiluted up to 1/32 were set up against Type II specific substance diluted 1/1,000 (Table VII).

As with the Type I specific substance and polyvalent serum (see Experiment 4) so here also the precipitin reaction between Type II specific substance and the particular specimen of polyvalent serum used reaches a high titre, slightly higher in fact in the serum dilutions for the polyvalent than for the homologous Type II serum. Here again, however, it can be shown by absorption that the reaction is due in large part to the non-specific precipitins in the polyvalent serum precipitating impurities, including the non-specific polysaccharide C, remaining in the type-specific substance.

Two samples of polyvalent serum were absorbed with homologous (Type II, Strain 442) and heterologous (Type I, Strain 454) whole organisms and set up against the dilutions of type-specific substance. Absorption with heterologous organisms removed a large amount of the antibodies from the serum, showing them to be group-specific; homologous absorption removed even more of the antibodies, these obviously being those against the type-specific substance.

Type II specific substance in a dilution of 1/100 gives a slight precipitate with antigonococcal serum and a still slighter reaction with antipneumococcal Type III serum. No reaction occurs in a dilution of 1/1,000. The reaction is due to the presence in the specimen of type-specific substance of some group- or non-specific fractions.

Chemical Analysis.—Crude specimens of Type II specific substance give a positive biuret test, a precipitate with 20 per cent trichloracetic acid and a weak Molisch reaction. The most purified specimen yet btained, which gives a precipitin reaction with homologous serum in dilution of 1/100,000, gives a positive biuret test and a marked recipitate with trichloracetic acid at 1/1,000 and a slight precipitate ith trichloracetic acid at 1/10,000. The Molisch reaction is positive at 1/100.

Type III

As has been mentioned above, the strain used in obtaining the Type II specific substance has been No. 302, secured from Dr. Sara Branam of the Public Health Service, Washington, as their most narowly specific Type III strain. Tested with the monovalent sera in use in this laboratory and using low dilutions in a 37°C. water bath, the strain gave an agglutination very similar to that given by any other Type I or Group I-III strain which has been isolated for some months and subcultured on artificial media (Table VIII).

TABLE VIII

Dilutions	Serum			
	I	II	III	IV
	No. 578	No. 551	No. 556	No. 535
1/10	++++	0	+++	0
1/25	++++	0	++	0
1/50	+++	0	++	0
1/75	++	0	+	0
1/100	+	0	+	0
Saline—negative				

Since this strain was not freshly isolated, it was to be expected that the yield of type-specific substance would be small, for this has been found to decrease in amount with the length of time the organism has been grown on artificial media (13). This fact explains, no doubt, some of the morphological changes which are characteristic of the transformation from "fresh" to "stock" cultures (14), and also the well recognized fact that stock strains usually show a loss of their type specificity as judged by the agglutination test (especially when that test is carried out at 55°C.). The total yield from this strain was 75 mg. of dried purified material. Of this, 55 mg. were obtained in the usual manner described above for isolation of type-specific substance. This specimen contained other substances which gave cross-precipitation with Type II and Type IV serum in the same way as the specimens of Type I and Type II specific substance gave cross-precipitation. The other 20 mg. were obtained from the nucleoprotein fraction or P which had been precipitated with 10 per cent acetic acid. This P fraction was found to contain not inconspicuous amounts of the type-specific fraction when it was tested with monovalent sera. It has been noted throughout the work that the nucleoprotein fraction contained varying amounts of type-specific substance which could be removed only with great difficulty. As a rule the amount was very

TABLE IX

	Serum			
	I	II	III	IV
	No. 578	No. 592[3]	No. 266	No. 577
No. 302 specific substance 1/250	$+++^{d}$	0	$+++^{d}$	0

TABLE X

Serum	Dilutions of type-specific substance						Serum	Dilutions of serum					
	1/250	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		1/1	1/2	1/4	1/8	1/16	1/32
Type I serum	$+++^{p}$	$+++^{p}$	$+^{p}$	0	0	0	Type I serum	$+++^{p}$	$+++^{p}$	$++^{p}$	$+^{p}$	0	0
Type III serum	$+++^{p}$	$+++^{p}$	$++^{p}$	0	0	0	Type III serum	$+++^{p}$	$+++^{p}$	$+++^{p}$	$++^{p}$	$+^{p}$	∓

TABLE XI

Serum..........................	I	I	III	III	I	III
Absorbed with....................	I	III	I	III	–	–
Type I specific substance 1/200	0	0	0	0	$++^{pd}$	$+++^{pd}$
Type III specific substance 1/250	0	0	0	0	$+++^{d}$	$+++^{d}$

small but in the present case it was quite considerable. As a consequence, it was precipitated with two volumes of ethyl alcohol and a scanty yield of type-specific substance obtained. The important feature about the type-specific substance secured in this way was the absence of impurities giving any significant cross-precipitations with Type II and Type IV sera (Table IX).

Titrations of the specific substance show that it behaves in a manner almost identical with that of the Type I specific substance. It gives a positive reaction with both Type I and Type III serum out to 1/10,000 but not to 1/100,000, and in dilutions of 1/1,000 it reacts with Type I serum diluted 1/8 and Type III serum diluted 1/32 (Table X).

Chemical analysis of the Type III specific substance fails to show any differences from the analysis of the Type I fraction. Thus, a purified specimen gives a positive Molisch reaction at 1/100, and a strong biuret test and a good precipitate with 20 per cent trichloracetic acid are demonstrable in a dilution of 1/1,000 though both are negative at 1/10,000.

An even more intimate relationship between the specific fractions is brought out by means of absorption experiments.

Experiment 9.—Samples of Type I and Type III serum were absorbed with whole organisms of both Type I and Type III strains. The organisms were left in contact with the serum for 2½ hours at 37°C. and 18 hours at the temperature of the ice box. These absorbed sera together with unabsorbed controls were then set up against specimens of specific substance from Strains 428 (Type I) and 302 (Type III). The results are shown in Table XI.

It will be seen that the absorption of either Type I or Type III serum by either Type I or Type III whole organisms removes completely the precipitins in these sera for both Type I and Type III specific substances. It has been demonstrated elsewhere (see Experiments 1 and 6) that, when strain of organisms and serum are heterologous, absorption with whole organisms helps to remove the non-specific antibodies in the serum but leaves untouched the heterologous precipitins for the type-specific substance. One can only conclude, therefore, from the above experiment (Experiment 9) that the type-specific substances isolated from the Type I and the Type III strains must be very similar in constitution if they be not identical.

body fluids of patients suffering from pneumococcal infections, especially in the cerebrospinal fluid of patients with pneumococcal meningitis (see Vincent and Bellot (5) and Dopter (6)). Boor and Miller (7), working with a non-protein fraction of the gonococcus, found a relationship between gonococcus, meningococcus and pneumococcus similar to that described below for the meningococcus fraction. In accordance with the term adopted for the similar fractions obtained from other microorganisms, this group-specific fraction from the meningococcus has been termed the "C substance."

As has been stated in the preceding paper of this series (1), the C substance may be rendered more pure by repeated alcohol precipitation. After the first two or three reprecipitations all of the precipitate is readily redissolved in distilled water, giving a clear, colorless solution.[1] A standard dilution of 1/100 may be kept in the ice box for a period of months without serious loss of serological activity, but the C substance keeps even better in the form of a dry powder which may be dissolved and used as required.

This carbohydrate fraction gives an abundant precipitate with some but not all samples of polyvalent antimeningococcal serum,[2] though it gives only a very slight precipitate with the monovalent rabbit sera prepared with freshly isolated strains in the manner described elsewhere (8).

Experiment 1.—A specimen of C substance obtained from Strain 23 (Type I) was set up, in a dilution of 1/100, against the four monovalent sera and a polyvalent antimeningococcal horse serum which previous experience had shown to possess a high precipitin titre (see Table I).

It will be seen that while the precipitate is heavy with the polyvalent serum, it is negligible with the monovalent sera prepared from

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[1] Although, in the description of the isolation of this C substance from the crude broth (1), the statement is made that on alcohol precipitation a heavy white precipitate settles out, this is not invariably the case and, especially when the broth has been concentrated, in its place there may appear a copious deposit of a brownish gum. The isolation of the C substance from this gum is a matter of much difficulty and recent work indicates that this group-specific fraction can be isolated more readily from extracts of organisms grown on solid media.

[2] For the specimens of polyvalent antimeningococcal horse serum the author is indebted to Dr. J. Zozaya and the New York State Department of Health.

fresh strains. It may be pointed out that the Type IV serum, in which the precipitate is heaviest, is prepared from a stock strain, no fresh strain being available.

The C substance from each of the three types reacts equally well with the polyvalent serum and absorption of the polyvalent serum with the C substance of one type removes the precipitins for the C substance not only of that type but of all the types.

Experiment 2.—Specimens of C substance from each of Type I, II and III strains in dilutions of 1/100 were set up against (1) unabsorbed polyvalent serum No. 3, and (2) polyvalent serum No. 3 absorbed twice with C substance from Strain 19, Type II. The ice box readings are given in Table II.

TABLE I

I	II	III	IV	Polyvalent		
No. 578	No. 592[1]	No. 266	No. 577	No. 3		
0	0	0	0	$+^{r}$	Immediate	Ring test
$\mp^{r}$	$\mp^{r}$	$\mp^{r}$	$\pm^{r}$	$+++^{p}$	1 hr.	
∓	∓	∓	±	$+++^{d}$	2 hrs.	
∓	∓	∓	±	$++++^{d}$	Ice box	

r = ring.
p = granular precipitate.
d = disc.

∓
±
+
++
+++
++++ = increasing amounts of precipitate.

The serological identity of the polysaccharide from the three different types is clearly shown by the results of this absorption test.

The C fraction is precipitated by other sera than polyvalent antimeningococcal serum. Antigonococcal serum and antipneumococcal Type III serum gave good reactions, while antipneumococcal Type II serum and one sample of antistreptococcal serum[3] gave slighter

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[3] For this and other antistreptococcal sera, the author is indebted to Dr. Rebecca Lancefield.

reactions. Antipneumococcal Type I sera, other specimens of antistreptococcal serum and sera prepared against the Gram-negative bacilli gave no reaction. The strong reaction with Type III antipneumococcal serum and the weak or negative reaction with Type II and Type I sera are in keeping with the fact that Type III serum always contains plentiful precipitins for the pneumococcus C substance, while sera prepared against Types I and II contain little. Table III shows the results of precipitin experiments between antimeningococcal polyvalent serum, antigonococcal serum, antipneumococcal sera and the active specimen of streptococcal serum and a specimen of meningococcus C substance (from Strain 23), diluted 1/100 and 1/1,000.

TABLE II

Type of C substance..	I	II	III
Strain No..	23	19	302
Unabsorbed polyvalent serum	++++^{d}	++++^{d}	++++^{d}
Polyvalent serum absorbed with Type II C	∓	∓	∓

TABLE III

Serum.........	Meningococcus		Gonococcus		Pneumococcus I		Pneumococcus II		Pneumococcus III		Streptococcus	
Dilutions of C substance.....	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}
23 C	++++^{d}	+++^{p}	+++^{p}	+^{p}	0	0	±	0	++^{p}	0	±^{p}	±

Conversely, polyvalent antimeningococcal serum and, to a lesser extent, antigonococcal serum will give a precipitate with the C substance of other microorganisms.

Experiment 3.—A specimen of pneumococcus C substance, highly purified and diluted 1/100, was obtained through the kindness of Dr. Forrest Kendall. This was set up against polyvalent antimeningococcal, antigonococcal and antipneumococcal Type III sera (Table IV).

The precipitates formed from the interaction of the antimeningococcal and antipneumococcal sera and the pneumococcus C substance were marked. That from the antigonococcal serum was less.

When these sera are absorbed with meningococcus C substance all the precipitins for the homologous C substance are removed, while those for the heterologous C substances are decreased but not altogether removed. Similarly, absorption of the sera with pneumococcus C substance removes the homologous precipitins completely but causes only a partial decrease in the heterologous precipitins.

Experiment 4.—Polyvalent antimeningococcal serum and Type III antipneumococcal serum were absorbed with dry meningococcus C substance (Strain 19, Type II), 10 mg. to 0.5 cc. of serum, for 2½ hours at 37°C. and overnight in the ice box. At the same time, similar quantities of the two sera were absorbed with 0.8 mg. of pure pneumococcus C substance (obtained through the courtesy of Dr. Walther Goebel). These absorbed sera were tested against meningococcus and pneumococcus C substance diluted 1/100 (Table V).

TABLE IV

Serum	Meningococcus	Gonococcus	Pneumococcus III
	No. 2	No. 011105-C	No. 81B-1
Pneumococcus C 1/1,000	$+++^{p}$	+	$++^{p}$

TABLE V

Serum		Polysaccharide	
		Meningococcus C	Pneumococcus C
Meningococcus serum	Unabsorbed	$++++^{d}$	$+++_{p}$
	Absorbed with meningococcus C substance	∓	$++^{p}$
	Absorbed with pneumococcus C substance	$+++^{d}$	0
Pneumococcus serum	Unabsorbed	$++^{p}$	$++_{p}$
	Absorbed with meningococcus C substance	0	$+\pm_{p}$
	Absorbed with pneumococcus C substance	$\pm^{p}$	0

These results would appear to indicate that the C substances obtained from different organisms, while very similar, are not identical chemically or immunologically. This differentiation of the two polysaccharides by means of the absorption test calls to mind a similar differentiation made by Avery, Heidelberger and Goebel (9) between the specific polysaccharides of Friedländer E and Pneumococcus Type II which had been found to resemble one another so closely. The case of the meningococcus and pneumococcus C substances, however, would seem to differ from that of the Friedländer E and Pneumococcus Type II specific substances in one particular. Avery, Heidelberger and Goebel found that absorption of Friedländer serum with the homologous organism removed all the precipitins and not merely those for the Friedländer E specific substance, but that absorption of this serum with the Pneumococcus Type II organisms removed only the precipitins for the Pneumococcus Type II specific substances. In the same way, homologous absorption of the pneumococcus serum removed all precipitins, while absorption with Friedländer organisms removed only the precipitins for Friedländer specific substance. In the case of the C substance of the meningococcus and pneumococcus, absorption of either serum with either C substance removes only the homologous precipitins and merely decreases, but does not remove, those for the polysaccharide other than that used in absorption. Since it was thought that the difference might be in the fact that in the latter case C substance alone was used for absorption while Avery, Heidelberger and Goebel had used whole organisms, the experiment with the meningococcus and pneumococcus sera was repeated using whole organisms for absorption.

Experiment 5.—Polyvalent antimeningococcal serum and Type III antipneumococcal serum were absorbed with whole meningococci. The growth used was a 16 hour culture of a freshly isolated Type II strain grown on blood agar plates, washed off in saline and centrifuged down, the serum being then added to the packed organisms which were mixed in with steady shaking of the tube. The absorbed sera and the same sera without absorption were now set up against solutions of meningococcus and pneumococcus C substance, both at 1/100. Table VI shows the results.

It will be seen that, in complete agreement with the work of Avery, Heidelberger and Goebel, the whole meningococci absorb from the antimeningococcal serum all of the precipitins, not only for the homologous but also for the heterologous C substance. On the other hand, absorption of the antipneumococcal serum with meningococci removes only the precipitins for the homologous C substance and leaves the pneumococcus C precipitins unaffected.

The C substance of the meningococcus will give a precipitin reaction in dilutions of one part in a million. This is a higher titre than is usually obtained with any of the type-specific substances. This may in part be due to the fact that the polyvalent antimeningococcal horse serum used in testing the C substance has of itself a higher titre than the monovalent rabbit sera used with the type-specific substances.

TABLE VI

Serum		Polysaccharide	
		Meningococcus C	Pneumococcus C
Meningococcus serum	Unabsorbed	+++^{p}	+++^{p}
	Absorbed with meningococcus whole organisms	∓	0
Pneumococcus serum	Unabsorbed	+±^{p}	++^{p}
	Absorbed with meningococcus whole organisms	0	++^{p}

TABLE VII

Dilutions of C substance....	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Dilutions of serum..	1/1	1/2	1/4	1/8	1/16	1/32
Polyvalent serum 1/1	++++^{d}	++++^{d}	++^{p}	+	∓	0	C substance 1/1,000	++++^{d}	+++^{d}	++^{p}	++^{p}	++^{p}	+^{p}

Experiment 6.—A specimen of meningococcus C substance (from Strain 23, Type I), purified by six reprecipitations with eight volumes of 95 per cent ethyl alcohol, was diluted from 1/100 to 1/10,000,000 and set up against undiluted polyvalent serum No. 3. Dilutions of the serum from 1/1 to 1/32 were set up against the C substance diluted 1/1,000 (Table VII).

It will be noticed also that C substance diluted 1/1.000 will give a precipitate in serum dilutions out as far as 1/32.

Chemical Analysis.—Purified specimens of C substance giving precipitin reactions out to 1/1,000,000 show a strong Molisch reaction at 1/100, a weak one at 1/1,000 and are negative at 1/10,000. Neither a positive biuret nor a positive trichloracetic acid test for protein can be obtained at 1/100.

The P Substance

This fraction—the P substance—, which is precipitated from the autolysate by means of 10 per cent acetic acid, appears as a greyish white flocculent precipitate. Unlike the C fraction and the type-specific substances which pack easily on centrifugation, the P substance is difficult to remove completely from the supernatant fluid and does not form a tight pack even after prolonged centrifugation. To some extent this appears to be due to the presence of mucoproteins which do not precipitate readily. The precipitate may be dissolved in N/100 Na_2CO_3 and then treated by repeated acetic acid precipitation and sodium carbonate resolution. After every precipitation there is a residue which will not pass back into solution. This residue decreases but slightly with further purifications, and must probably be regarded as protein denatured during the process of purification. It is fully realized that the sample of so called nucleoprotein with which one finishes may well be somewhat different from that occurring *in vivo*, or even on first precipitation, but one can conclude that whatever changes have taken place have been unable to alter to any great extent the reactive properties of the substance which still precipitates in a characteristic manner with the appropriate serum. It may also be stated at this point that it is doubtful whether any of the protein material obtained in this, the accepted method, from microorganisms consists of nucleoprotein, despite the common appellation of nucleoprotein applied to this material in the literature. Certainly, very little can be regarded as nucleoprotein and the bulk consists of other conjugated proteins which preliminary work suggests can be separated one from another by various manipulations. Only very small amounts of carbohydrate are present even in the first crude specimens of P substance. A constant feature, however, is the presence of type-specific substance in the first crude specimens and persisting even for the first few purifications. This fact is clearly demonstrated when crude specimens of P substance from the different types are tested with monovalent and polyvalent sera (Table VIII).

It will be noted that the crude P fraction, when tested with monovalent serum, reacts only with sera homologous to the type from which it is derived. The type-specific substance to which this reaction is due can be removed by purification, giving a solution which still reacts strongly with the polyvalent antimeningococcal serum but no longer with any of the monovalent sera. Where the type-specific fraction is present in sufficiently large amounts it can be recovered by precipitation with two volumes of ethyl alcohol and the specimen thus obtained is much purer than that obtained by the usual method, since it contains only undemonstrable amounts of C substance, if any at all.

TABLE VIII

Serum	I	II	III	IV	Polyvalent No. 3
No. 428 Type I P^2	+	0	+	0	$++++^{p}$
No. 31 Type II P^2	0	±	0	0	$++++^{p}$
No. 302 Type III P^2	$++^{p}$	0	$+++^{p}$	0	$++++^{p}$

TABLE IX

Serum..........................	Meningococcal		Gonococcal		Pneumococcal	
Dilutions of P substance..........	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}
	$++++^{p}$	$++^{p}$	$++^{p}$	±	$++^{p}$	0

Like the C substance, the protein fraction reacts with both antigonococcal and Type III antipneumococcal sera (Table IX), but not with any of the sera tried; *viz.*, antistreptococcal sera and sera prepared against the Gram-negative bacilli. This is in keeping with the work of Boor and Miller (7) who showed that the nucleoprotein which they obtained from the gonococcus was likewise not species-specific.

The P fraction will give a precipitin reaction in a dilution of 1/100,000 but not in 1/1,000,000, which is lower than that obtained with the C substance when the same polyvalent antimeningococcal serum is used.

Experiment 7.—A specimen of meningococcus P substance (from Strain 428, Type I), precipitated by three reprecipitations with 10 per cent acetic acid, was set up in dilutions of from 1/100 to 1/10,000,000 against polyvalent serum (No.

3) undiluted. Dilutions of the serum up to 1/32 were set up against a 1/1,000 solution of P substance (Table X).

P substance at 1/1,000 reacts with a serum dilution of 1/16 but not one of 1/32.

Chemical Analysis.—Purified specimens of P substance giving precipitin reactions out to 1/100,000 give a negative Molisch reaction at 1/100. Both biuret test and precipitate test with 20 per cent trichloracetic acid are positive at 1/10,000 dilution, but not at 1/100,000.

All of the specimens of P substance which have been tested have proved to be highly toxic for rabbits and, to a much less degree, for mice. The toxicity of various preparations varies to some degree, but the majority have a strength such that 1 to 3 mg. injected intravenously will kill a 2,000 gm. rabbit within 6 hours. The toxic factor is relatively stable and the suspension of P substance can be stored in the ice box over a period of weeks without showing any great loss in toxicity. Specimens of P substances, besides being toxic, are strongly antigenic and will evoke a serum which, in addition to having a high precipitin titre for the meningococcus P substance, will protect against many lethal doses of the toxic factor contained in the P substance. It must be stated here that it is by no means certain at the moment that the P substance and the toxic factor are one and the same thing. Investigations are at present under way concerning this particular point.

TABLE X

Dilutions of P substance....	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Dilutions of serum.....	1/1	1/2	1/4	1/8	1/16	1/32
Polyvalent serum 1/1	$++++^{p}$	$++^{p}$	±	∓	0	0	Protein 1/1,000	$++^{p}$	$++^{p}$	+	±	∓	0

DISCUSSION

Three substances, which react in a specific manner with appropriate sera, have been isolated from the meningococcus. Of these substances, the first or type-specific substance has been described in a previous paper (1), while the other two, which are group-specific, have been described above.

The method of isolation used for these substances has been chiefly that of prolonged growth of the organisms in fluid media with autolysis occurring *pari passu* with this growth. It is realized that such prolonged cultivation in fluid media must give rise to many substances which are products of the bacterial metabolism or are formed as a result of the interaction of the organism and its pabulum, the surrounding medium. In such a case, doubt may exist as to whether the serologically active substances are preformed in the organism or are not rather the result of interaction of meningococci and media. In order to surmount this objection, other methods of obtaining the serologically active substances have been used. The organisms have been grown on solid media for short periods of time, *i.e.* under 20 hours, and then washed off. The bacterial growth obtained in this manner has been extracted with saline, weak alkalies or acids, and the extracts so obtained have been subjected to methods much the same as those applied to the broth cultures. By such means it has proved possible to isolate three serologically active substances which now behave in like manner to those obtained from the broth. Zozaya and Wood (2) and Zozaya (3) used similar methods for the isolation of group-specific protein and polysaccharides of the meningococcus. The method which calls for the use of solid media has not been adopted as a routine because of several complicating factors. Foremost of these is the influence of the agar. As Sordelli and Mayer (10) and others have pointed out, saline solution or any other solvent brought into contact with agar, dissolves up some fraction present in the agar, which will now give a precipitin reaction with sera produced by the inoculation of vaccines prepared from bacteria grown on agar plates. Since all sera used in the present work are prepared from agar-grown vaccines, it is clear that complications will arise in the use of organisms grown on media with this basis for the preparation of the serologically active substances. Apart from this is the fact that growth in broth is a far less laborious method of preparing the crude substances and one which gives a far greater yield.

The fact that sera containing antibodies for these chemical substances isolated from the broth cultures can be obtained by the inoculation of young and presumably intact cultures of meningococci would lead to the assumption that such chemical substances, or others perhaps more complicated but containing the essential radicals, must be present in the intact organism and not be a product merely of metabolic activity. This assumption is further supported by the fact that similar substances may be obtained from young organisms not allowed to autolyze but killed and extracted immediately, as has been pointed out above.

It is believed that these fractions bear a relation to certain of the facts recognized in connection with the meninogococcus itself and with the reaction of the host to invasion with this organism. It would seem clear that the type specificity of the meningococcus, which by the use of a modified agglutination technique (8) seems to be a definite and distinct entity, depends, at least in part, on the presence within the organism of the appropriate member of the type-specific substances described and, moreover, that the specificity of agglutination and other serological reactions depends on the interaction of that substance and the corresponding antibody in the serum.

The clear-cut type specificity of the serological reactions is best seen with sera prepared against freshly isolated strains of meningococci. Sera prepared from stock strains tend to give group-specific as well as type-specific reactions, especially when lower serum dilutions are used. This would appear to be due to the fact that, as has been shown by Petrie (11) and in this laboratory, strains of meningococci when repeatedly subcultured on artificial media lose much of the type-specific substance which characterized them in the freshly isolated state; indeed, several of the differences to be noted between stock and fresh strains may be due to this fact. When such stock strains are used in the production of serum, the type-specific antibodies are formed only slowly and prolonged immunization is required to obtain a serum of sufficient antibody content. The serum produced by such a period of immunization is one of broad non-specificity giving cross-reactions, at least in lower dilutions. It would seem that the animal, while reacting to the type-specific substance, has formed antibodies also against the group polysaccharide and protein (C and P) fractions. The presence of antibodies for these group substances in the serum will account for the cross-reactions. The animal appears to react more readily to the type-specific than to the group substances, for it is a fact that antibodies against the former appear sooner in the serum than those against the latter. Why this should be is at present not clear. It may be that the type-specific substance lies near the surface of the organism, but it should be emphasized that the meningococcus apparently does not possess a capsular structure of type-specific substance such as is presumed for the pneumococcus. It is nevertheless a fact that when fresh strains with plentiful type-specific substance are used, sera with a sufficiently high titre of type-specific antibodies but with low titre of group-specific antibodies can be obtained promptly. On the other hand, to obtain a serum with group-specific antibodies, using whole organisms, it is necessary to immunize over a period of many months (12).

The antigenic structure of the organism probably accounts for certain other peculiarities of the agglutination reaction. It has been shown (8) that agglutination at 37°C. is more satisfactory than that at 56°C., owing to the absence of cross-reactions. It may be said in parenthesis that this applies only to the use of sera prepared with fresh strains (anti-S sera), since sera prepared with stock strains seem to be too low in type-specific antibodies to react at the lower temperature. Using anti-S sera in low dilutions, the reaction is rapid and clear-cut at 37°C.; at 56°C., however, cross-agglutination tends to obscure the results and the suspended organisms appear to lose their type specificity. It can be shown that warm saline, in which meningococci have been suspended for some hours and then centrifuged out, contains type-specific substance. One may therefore suppose that agglutination at 56°C. is unsuitable because at this temperature the type-specific substances diffuse out readily into the surrounding fluid and the group substances, either by being exposed or by becoming predominant, react with their appropriate antibodies to produce non-specific agglutination.

Both type-specific and group substances have been demonstrated in the body fluids of those suffering from meningococcus infections. They can be shown to exist in the spinal fluid in cases of meningitis due to this organism, where they give a precipitin reaction with anti-S monovalent sera (13). As might be expected, the strength of precipitin reaction obtained is directly dependent on the numbers of meningococci in the spinal theca which have undergone autolysis, and thus, more indirectly, on the total number of infecting organisms. Where this is very low, the precipitin titre is also very low or absent; moreover, treatment with antimeningococcal serum tends to diminish or remove entirely the precipitinogens, so that a reaction is frequently no longer to be obtained once therapeutic treatment has been commenced.

SUMMARY

Two group-specific substances, one a polysaccharide and the other a protein, have been isolated from the meningococcus and their serological properties are described. A discussion of these substances, together with the type-specific fraction, is given, and a relationship of these substances to certain serological phenomena is suggested.

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STUDIES ON MENINGOCOCCUS INFECTION

V. The Presence of Meningococcus Precipitinogens in the Cerebrospinal Fluid

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In 1909, as a result of the published work of Bruckner and Cristeanu (1) and Dopter and Koch (2) on the occurrence in antimeningococcal serum of precipitins for extracts of the meningococcus, Vincent and Bellot investigated the spinal fluid of patients suffering from meningococcal and other forms of meningitis (3, 4). They showed that polyvalent antimeningococcal horse sera, prepared by the injection of whole organisms or of autolysates of the organisms in distilled water, gave positive results in the majority of cases of meningococcal meningitis. Normal cerebrospinal fluid and fluid from other forms of meningitis, with the occasional exception of that due to the pneumococcus, were negative, but spontaneous precipitation might occur if the spinal fluid were not freshly drawn. Positive reactions could be obtained 11 hours after the onset of the disease and were found as late as 50 days, but fluid obtained by delayed puncture, even in untreated cases, might fail to show the reaction. No reaction was found in cases which had received serum intrathecally. The reaction was carried out by adding 2 to 5 drops of serum to 50 to 100 drops of fluid cleared by centrifugation. The tubes were kept at 37° or 50°C. for from 8 to 40 hours. In the same year these results were confirmed by Lemoine, Gaehlinger and Tilmant (5), Louis (6) and Letulle and Lagane (7). The latter authors noted especially the fact that spontaneous clouding of the cerebrospinal fluid might occur, but it is noteworthy that all these tests were carried out at 52°C. for from 8 to 10 hours. Bruynoghe (8) obtained seven positive reactions out of twelve known meningococcal spinal fluids. The fact that a larger percentage was not positive and further that a reaction was obtained with the spinal fluid from a case of poliomyelitis caused him to doubt the value of the test. Worster-Drought and Kennedy (9) obtained twelve positive results in twenty-eight known cases. Dopter (10) found some use for the test but noted spontaneous clouding of the spinal fluid (his tests were carried out at 55°C.), positive results with normal horse serum and reactions in spinal fluid from meningitis due to organisms other than the meningococcus; *i.e.*, pneumococcus and the tubercle bacillus. He also found it negative in some definite cases of meningococcus meningitis. In a recent communication (11) Kreidler and Murphy have reported results which agree very largely with those already given. Their tests were carried out at room temperature, using equal parts of serum and spinal fluid. Normal spinal fluids gave no reaction, and none was found to occur between meningococcal spinal fluid and antipneumococcal serum or between pneumococcal spinal fluid and antimeningococcal serum.

All the authors already quoted have used polyvalent antimeningococcal serum and have made no attempt to utilize the precipitin test on the cerebrospinal fluid as a means of typing the organisms responsible for the infection. Marie (12), however, showed that cases of meningitis due to the meningococcus can be separated from those due to the parameningococcus by the use of monovalent sera prepared against these types. No other papers bearing on this point have been found.

The demonstration of type-specific substances for Type I-III and Type II strains of meningococcus and the production of monovalent rabbit sera which have a high titre of precipitins for these type-specific substances but a very low titre of precipitins for the non-type-specific (C and P) fractions (13, 14), has led to the investigation of cerebrospinal fluid from cases of meningococcal meningitis in order to ascertain whether this contains type-specific precipitinogens. A preliminary paper has offered evidence that such is indeed the case (15). The present communication deals with the matter more comprehensively.

Material and Technique

Spinal fluid was obtained from cases of known meningococcal meningitis and from other cases of meningitic infection or disease of the central nervous system. The precipitin test was carried out with equal parts of undiluted serum and spinal fluid, cleared by centrifugation. The tubes were kept for 2 hours at 37°C. and for 15 to 18 hours at ice box temperature. Both the ring method and the results obtained by mixing serum and spinal fluid were used for reading.

0.1 cc. of each of the four type sera was placed in a small precipitin tube. 0.1 cc. of the spinal fluid was carefully layered on top of each of the sera so that a clear line of junction was formed between the two fluids. In this manner any precipitate formed was seen as a distinct white ring at the point of junction and could be made out with ease. A reading was made immediately and the tubes were then placed in the 37°C. water bath. At the end of 1 hour a further reading was made and the tubes were agitated in order to mix the two fluids thoroughly. The tubes were again returned to the water bath and read at the end of another hour. They were then placed in the ice box overnight, at the end of which time the final reading was made.

When the work was commenced, the cerebrospinal fluid was tested with monovalent rabbit serum prepared against all four types of meningococcus. Since, however, the Type IV serum had been prepared against a stock Type IV strain, no freshly isolated strain of this type having been obtainable, this serum, unlike the others, contained a moderately large amount of the non-type-specific precipitins and gave, as was to be expected, cross-reactions. In the more recent tests the Type IV serum has been omitted. Of all the specimens of cerebrospinal fluid investigated, only those due to the meningococcus are recorded and discussed here from which the strain of organism involved could be grown and typed by means of the agglutination test.

RESULTS

Twenty-three cerebrospinal fluids have been obtained from cases of cerebrospinal meningitis in which the corresponding strain of organisms was also recovered. In addition to these, eight fluids from other forms of meningitis or disease of the central nervous system have been tested. The results of the precipitin tests are given in Table I, and the type as ascertained by means of the agglutination test is given in a separate column of the same table.

In examining the results obtained from cases of meningococcal meningitis it will be noted that, if the results with the Type IV serum be excluded, those obtained by means of the precipitin reaction correspond with those found on agglutination and that there is on the whole no cross-precipitation. The apparent cross-precipitation between Type I and Type III sera must be viewed in a different light. As has been pointed out elsewhere (13), there is no demonstrable qualitative difference between the Type I and Type III strains obtainable at the present time as regards their type-specific substance. Cross-precipitin and cross-agglutinin reactions are therefore to be expected, and the custom has been adopted of referring to these strains as belonging to the I-III group rather than to either Type I or Type III. The cross-precipitation obtained with the Type IV serum is most certainly due to the presence in the spinal fluid of non-type-specific substances (both C and P fractions) produced by the autolysis of the meningococci. One must assume that the presence of type-specific substance in the centrifuged and cleared specimens of cerebrospinal

TABLE I

Cerebrospinal fluid strain No.	Type I serum	Type II serum	Type III serum	Type IV serum	Agglutination	Notes
23	$++\pm^{pd}$	0	$++^{p}$	$\pm^{p}$	I-III	
28	$+^{p}$	0	$+^{p}$	∓	I-III	
425	$+++^{p}$	0	$++^{p}$	+	I-III	
449	∓	0	±	±	I-III	
451	$++^{pd}$	0	$++\pm^{pd}$	0	I-III	
460	0	0	0		I-III	No serum treatment. Smear showed only very few meningococci
462	∓	0	±		I-III	
465	$+\pm^{p}$	0	$+^{p}$		I-III	
473	0	0	∓		I-III	? after start of intrathecal serum therapy
484	±	0	±		I-III	
488	$++^{p}$	∓	$+^{p}$		I-III	Before start of intrathecal serum therapy
488	±	0	±		I-III	After 1 dose of intrathecal serum
489	$++^{p}$	0	$++^{p}$		I-III	
490	±	0	±		I-III	
436	0	±	0	∓	II	
464	0	$++^{pd}$	0	0	II	
467	0	$+^{p}$	0		II	
477	0	±	0		II	Before start of intrathecal serum therapy
477	0	0	0		II	After start of intrathecal serum therapy
481	0	0	0		II	After start of intrathecal serum therapy. Very few meningococci in smear
486	0	0	0		II	? after start of intrathecal serum therapy
487	0	0	0		II	Before start of intrathecal serum therapy. Smear showed very few meningococci

429	0	+	0	0	II	Before start of intrathecal serum therapy	A	0	0	0		*Strep. viridans meningitis*
429	0	0	0	0	II	After start of intrathecal serum therapy	B	0	0	0		Pneumococcus I meningitis
491	$+^{p}$	0	±		I-III	Before start of intrathecal serum therapy	C	0	0	0		Pneumococcus III meningitis
492	$++^{p}$	0	$+^{p}$		I-III	Before start of intrathecal serum therapy	D	0	0	0		Pneumococcus X meningitis
492	0	0	0		I-III	After start of intrathecal serum therapy	E	0	0	0		Encephalitis
							F	0	0	0		Tuberculous meningitis
							G	0	0	0		Tuberculous meningitis
							H	0	0	0		Subarachnoid haemorrhage

fluid is due to autolysis of the organisms, and it is evident that the type-specific substance cannot be set free without the liberation at the same time of certain amounts of both non-specific carbohydrate C substance and group-specific protein P. The Type IV serum, being prepared with a stock rather than a freshly isolated Type IV strain and containing antibodies for these fractions, will give cross-precipitation while the other monovalent sera do not. In fact, in all of the later tests the use of Type IV serum has been omitted, and this step can be justified by the fact that at the present time very few, if any, cases of meningitis due to this type are to be found and none has come to the notice of this laboratory in 2½ years.

Five spinal fluids from cases of meningococcus meningitis, which fail to give any appreciable precipitin test even after standing overnight in the ice box, are shown in the table. Of these, two belong to the Group I-III and three to Type II. Now it has come to light in the course of this investigation that, as was to be expected, those spinal fluids in which smears show meningococci to be plentiful contain precipitinogens in larger amounts, while small numbers of organisms are related to a low titre of precipitinogens. Thus it has been found that in spinal fluids, such as Nos. 436, 449, 460 and 487, in which the precipitin reaction is weak or absent, there is a corresponding scanty number of organisms to be found on examination of a smear. On the other hand, in cases such as Nos. 425, 451, 464 and 489 in which the precipitin reaction is strong, meningococci are present in large numbers and must produce a rich autolysate. It is only to be expected that at least until sera with a high precipitin titre are available a certain number of negative tests must occur.

Quite apart from this is the relationship of the time at which the fluid was drawn from the body to the commencement of intrathecal serum therapy. It is important that the cerebrospinal fluid for precipitin tests be obtained before intrathecal serum treatment has been commenced, or early in the course of such treatment. Spinal fluids which have been positive before serum therapy rapidly become negative and although weak reactions may be obtained in the first 1 or 2 days after the serum injection has been begun, further injections of serum will produce a spinal fluid which is altogether negative. This is clearly demonstrated by fluids Nos. 429, 477, 488 and 492 which, positive before treatment, become negative or very weak in precipitinogens after intrathecal serum inoculation. One must suppose that the antibodies in the serum unite with the type-specific substance in the body and remove it from solution. Of the negatively reacting fluids, one, No. 481, was certainly obtained after serum treatment had been begun, while two others, No. 473 and No. 486, were believed to have been drawn off after one treatment, though this point is not certain. These two factors—the lack of sufficiently sensitive sera and the failure to obtain all fluids before treatment was begun—will explain all the negative results or failures which were encountered. If the three fluids, Nos. 473, 481 and 486, be omitted, it will be seen that only two negative results were obtained in twenty fluids, that is 10 per cent, and this figure could certainly be reduced with the use of higher titre sera.

TABLE II

Cerebrospinal fluid strain No.	Type I serum	Type II serum	Type III serum	Type by agglutination
23	++^{p}	0	+	I-III
28	+	0	±	I-III
465	+	0	+^{p}	I-III
484	±	0	±	I-III
488	+^{p}	0	±^{p}	I-III
489	+^{p}	0	±	I-III
436	0	±	0	II
467	0	+^{p}	0	II

The eight spinal fluids from diseases of the meninges or central nervous system other than meningococcal meningitis fail to give any precipitation with the monovalent sera. Tests with polyvalent antimeningoccal horse serum rich in precipitins for both C and P fractions have been made and it has been found that fluids from cases of pneumococcal meningitis will occasionally give weak precipitin reactions. This is in keeping with the work of other investigators (3, 4, 10) and with the fact that the group-specific substances of the meningococcus, especially the C substance, are related to those of other organisms including the pneumococcus.

It is believed that the observations given in this paper may be of practical importance. They offer a rapid method of determining to which type of meningococcus a given case of meningitis is due. In the past, one of the chief criticisms against the use of monovalent and in favor of the use of polyvalent therapeutic antimeningococcal serum has been the delay of 2 or more days necessary before the type of organism can be ascertained by means of the usual method of agglutination. During this period one must perforce give polyvalent serum, if any serum therapy at all be attempted. In Table I the readings given are those made 17 to 20 hours after the tests were set up and therefore could have been made 17½ to 20½ hours after withdrawal of the fluid from the body. Such a period of time is not, however, essential and the majority of spinal fluids will allow of a diagnosis on removal of the tubes from the water bath at the end of 2 hours. Table II shows the readings on eight of the specimens of spinal fluid made at the end of 2 hours at 37°C.

In all of these a diagnosis could have been ventured at the end of 2 hours, that is 2½ hours approximately after the fluid was withdrawn from the body. In many cases, indeed, the precipitin reaction is immediate and is sufficiently strong to allow one to make a diagnosis directly spinal fluid and serum come into contact.

In correspondence with the long period of time over which solutions of type-specific substance isolated *in vitro* will keep their full reactivity in the ice box, specimens of spinal fluid, if stored at 0°C. will continue to give a precipitin reaction over many months. Thus, spinal fluids No. 23 and No. 28, 14 and 12 months respectively after they were drawn, gave precipitin reactions as strong as when they were first obtained.

SUMMARY

Precipitin tests, carried out on the cerebrospinal fluid from cases of meningococcal meningitis with monovalent sera, demonstrate the presence in that fluid of type-specific precipitinogens of the meningococcus. Negative results are secured when the spinal fluid is obtained after the commencement of intrathecal serum treatment and also occasionally when the numbers of infecting organisms are very small. The reaction offers an easy and rapid method of ascertaining to which type of meningococcus a particular case of meningitis is due,

and facilitates the immediate use of monovalent therapeutic antimeningococcal serum. Typing by means of the precipitin reaction can be confirmed by agglutination of the strain of organism responsible for the infection, if such strain be isolated. Confirmation by means of agglutination has been possible in all the cases discussed in this report. Spinal fluids from other diseases of the meninges and central nervous system fail to give any precipitin reaction with the monovalent sera.

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EXPERIMENTAL AND STATISTICAL EVIDENCE OF THE PARTICULATE NATURE OF THE BACTERIOPHAGE*

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In spite of the fact that the bacteriophage is invisible under the microscope and passes readily through porous filters, most observers seem to be agreed that it is distributed throughout a medium as some kind of a particulate entity. Whether it is of an organized corpuscular

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CORRECTION

On pages 202 and 205, Vol. 58, No. 2, August 1, 1933, the cuts for Text-figs. 2 and 3 should be interchanged. The cut on page 202 is Text-fig. 3, and should appear on page 205. The cut on page 205 is Text-fig. 2, and should appear on page 202. The legends should remain on the pages where they now appear.

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containing a given number of bacteria can be tested experimentally and expressed mathematically. Eminent mathematicians have abundantly confirmed and extended the McCrady concept, discovering finally that it is but a special case of Poisson's law of small chances. It states simply that the concentration of particles in a volume is equal to the negative natural logarithm of the proportion of volumes in which particles are absent. Wells and Wells (4, 5) have discussed the application of this law to the bacteriological system of geometric dilution

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* Laboratory facilities for carrying on an investigation involving bacteriophages, of which this paper is a by-product, are being furnished through the courtesy of Dr. W. G. Smillie.

series, and have offered a simplified index which is consistent with the theory and adapted to practical use. In the application of Poisson's law to the bacteriophage, the logic is reversed to secure a statistical approach to the question as to whether or not the bacteriophage satisfies the conditions required if it were particulate. This paper records the results of experiments made to test the hypothesis.

Bronfenbrenner (6) set up an experiment in which there were 20 tubes in each of two series. In one series the dilution was 1 × 10^{-10} and four tubes (20 per cent) failed to show lysis. In the other series the dilution was 1 × 10^{-11} and 19 tubes (95 per cent) failed to show lysis. The variation he accounted for on the law of chance that some of the tubes in the first series would receive more than their share of the particles while others would receive less, which in four tubes happened to be none. In the same way in the second series, which usually contains no bacteriophage, an occasional tube will receive a phage particle and show lysis.

Clark (7) discussed the theory of serial dilutions and stated that Bronfenbrenner's estimate, that 85 per cent of parallel titrations of the same bacteriophage would show lysis in the same tube of the series, did not check closely with what he calculated to be the most probable per cent of agreement that might be expected, which he claimed should be 60 per cent. He noted that Brofenbrenner's estimates were more in the category of impressions and not computations from actual records. Likewise he did not feel justified in offering this lack of agreement as evidence against the particulate nature of the bacteriophage.

If the bacteriophage is actually particulate, observations must approximate statistical requirements more closely than would be indicated in Clark's paper. To determine whether or not the required conditions are fulfilled, experiments were set up in which the number of observations would be great enough to be statistically significant.

In Experiment I five dilutions (1 × 10^{-8}, 1 × 10^{-9}, 1 × 10^{-10}, 1 × 10^{-11} and 1 × 10^{-12}) of a bacteriophage active against the Hiss Y dysentery bacillus were made and each flask was seeded with the Hiss Y dysentery bacillus. Each dilution was then distributed in 5 cc. quantities into 160 sterile tubes and 18 hours later the number of tubes in which lysis failed to occur was recorded. All the tubes in the 1 × 10^{-8} series showed lysis. The per cent failing to show lysis in the other four dilutions are shown in Table I. The labor of distributing the dilutions in long series was greatly lessened by using special racks holding 10 tubes each which eliminated the necessity of handling cotton plugs. These racks were generously loaned to us by the Department of Sanitary Engineering of Harvard University.

In each of the three succeeding experiments the dilutions were made closer together and were chosen nearer and nearer the center of the curve, where observations tend to be more uniform than at the ends of the curve. The results of these experiments are likewise recorded in Table I.

TABLE I

Comparison of Theoretical and Observed Values

Dilution of bacteriophage	Tubes failing to show lysis							
	Experiment I (160 tubes in series)		Experiment II (160 tubes in series)		Experiment III (100 tubes in series)		Experiment IV (150 tubes in series)	
	Calculated*	Observed	Calculated*	Observed	Calculated*	Observed	Calculated*	Observed
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
10×10^{-10}	0.0	1.8						
5×10^{-10}			0.05	2.2				
3×10^{-10}					1.8	2.0		
2×10^{-10}					6.9	14.0		
1.58×10^{-10}			17.4	11.4				
1×10^{-10}	28.9	33.1			26.3	31.0		
0.8×10^{-10}							31.7	31.9
0.7×10^{-10}							36.8	37.8
0.6×10^{-10}							42.2	41.8
0.5×10^{-10}			57.5	58.7	51.3	47.0	48.6	47.9
0.4×10^{-10}					58.6	54.0	56.3	58.6
0.3×10^{-10}					67.1	64.0	65.3	65.3
0.2×10^{-10}					76.9	75.0	75.0	76.0
0.158×10^{-10}			83.9	85.0				
0.1×10^{-10}	88.3	85.6			87.5	88.0	86.7	84.7
0.05×10^{-10}			94.6	93.7				
0.01×10^{-10}	98.7	98.1						

* Estimated on the assumption that dilution 1×10^{-10} contained the following number of phage particles per distributed volume: Experiment I, 1.244; Experiment II, 1.105; Experiment III, 1.337; Experiment IV, 1.439.

Various workers have discussed methods of determining the most probable number of particles in a suspension when the number of tubes of various dilutions which fail to receive a particle is known. Given the percentage of negative tests in various dilutions, the probable number of particles in each dilution can be selected from tables, such as those recently published by Halvorson and Ziegler (8).

The most probable numbers of phage particles were read from this table by looking up the values of e^{-x}, corresponding to the percentage of tubes negative for each dilution. This was done for each of the experiments. The values for the various dilutions in Experiment IV are recorded in Table II. The values were then reduced to a common base (1×10^{-10}) by dividing by the factor necessary to make the dilution equivalent to that of the base. It will be seen that the figures in this column fall quite closely together, the lowest being 1.300 and the highest 1.650 phage particles per distributed volume. If a mean of these values is calculated, it gives the most probable number of phage particles in dilution 1×10^{-10} when all the dilutions are given equal weight. This mean in Experiment IV is 1.439. (In the other experiments the values are: Experiment I, 1.244; Experiment II, 1.105; Experiment III, 1.337.

TABLE II

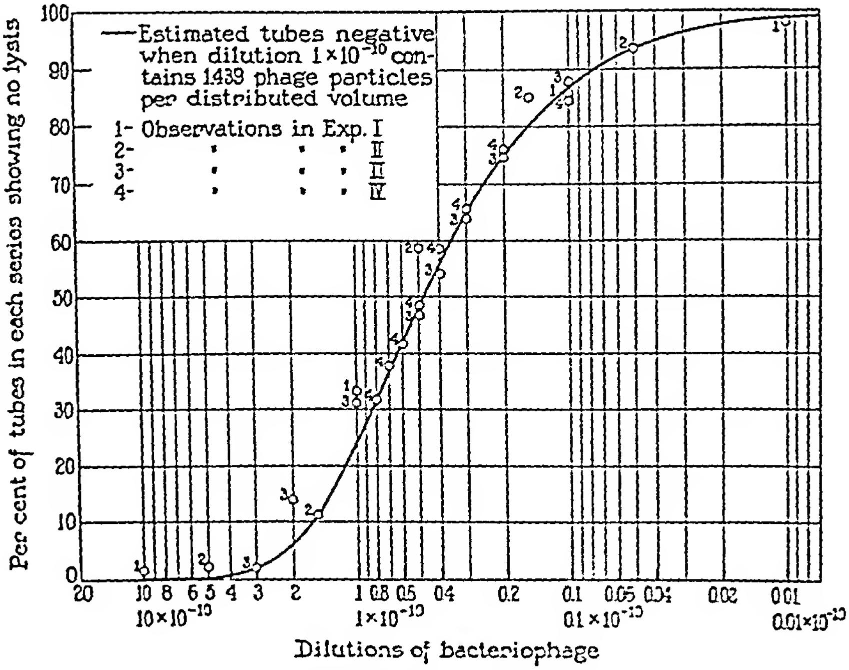
Calculation of the Most Probable Number of Phage Particles in a Suspension (Experiment IV)

Dilution of bacteriophage	Tubes negative	Most probable number of phage particles per distributed volume*	Values in previous column reduced to common base (dilution 1×10^{-10})†
	per cent		
0.8×10^{-10}	31.9	1.150	1.440
0.7×10^{-10}	37.8	0.975	1.300
0.6×10^{-10}	41.8	0.870	1.430
0.5×10^{-10}	47.9	0.745	1.470
0.4×10^{-10}	58.6	0.535	1.340
0.3×10^{-10}	65.3	0.425	1.417
0.2×10^{-10}	76.0	0.275	1.375
0.1×10^{-10}	84.7	0.165	1.650
			8)11.512
			1.439

* Values taken from table published by Halvorson and Ziegler, mentioned in the text.

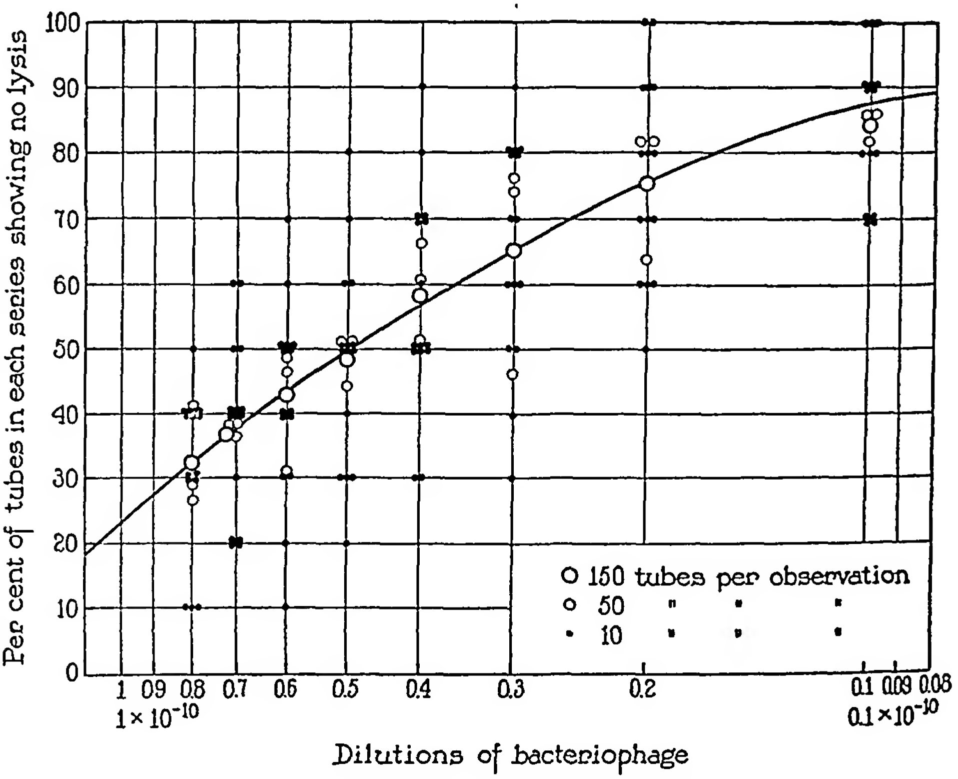
† Obtained by dividing 1.150 by 0.8, 0.975 by 0.7, etc.

Using these means we can now go back to the table of Halvorson and Ziegler and read off the number of tubes which would be expected to be negative if various dilutions of the phage were set up against the homologous organism. A number of such values are recorded in Table I. In reading across this table, it will be seen that in most instances the observed values are fairly close to the calculated values. Such discrepancies as are noted are due to experimental errors and to chance variation. The variations due to chance could be still further reduced by increasing the number of tubes in each series to 500 or 1000, but the values obtained seem to be accurate enough for the present discussion.



GRAPH 1. This figure shows how closely the observed per cent (represented by circles) of tubes failing to show lysis in various dilutions of bacteriophage come to the theoretical curve (represented by the heavy line).

In order to be able to see at a glance how close the agreement is, a curve represented by the heavy line was plotted in Graph 1 from the calculated values for Experiment IV. The actual observations were shown as circles with figures to indicate to which experiment they belonged. The circles follow the curve closely. For the sake of simplicity the curves for the other three experiments were omitted and all of the observed values are compared to the one curve. The other three have the same shape but fall slightly higher than that of Experiment IV, which explains why so few of the observations of the first three experiments are below the line. Plotted against their own curve some observations would be above and some below the line.



Graph 2. This figure demonstrates clearly that by decreasing the influence of chance through increasing the number of tubes in a series the agreement between theoretical and observed values becomes closer and closer (graph made from figures of Experiment IV).

In order to show that it is necessary to make a large number of observations before conclusions can be drawn, Graph 2 was made. In this graph the theoretical curve and the actual observations in Experiment IV were plotted. Each of the large circles represents 150 tubes, as in Graph 1. By breaking up each series into three groups of 50 tubes, we can observe how much farther away from the curve the observations fall because of the decrease in the number in each series. The smaller circles represent observations of 50 tubes each. Breaking up each series still farther, we have 15 groups of 10 tubes each and the dispersion is still greater, as shown by the black dots. It is easy to see that if we had used only 10 to 20 tubes to a series there might have been some chance of the figures being so irregular as to cause doubt concerning whether there was any agreement between theoretical and observed values. Conversely, if we had used 1000 tubes in a series, the parallelism would have been more striking, because of the decrease in the influence of chance.

These observations point strongly to the particulate nature of the bacteriophage. It is difficult otherwise to explain the closeness of fit, over so wide a range, of observed values with values computed from laws characteristic of particles in suspension. The bacteriophage fulfills this important requirement of particulate entities in that it does not appear to exist in a medium except in association with something which is particulate. Speculation on how such an association would be possible without the bacteriophage being in itself particulate is not the purpose of this paper.

SUMMARY

Experimental evidence is brought forward to show that the bacteriophage obeys very closely the laws of chance distribution of particles in suspension.

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FUNCTION OF THE GALL BLADDER EPITHELIUM AS AN OSTEOGENIC STIMULUS AND THE PHYSIOLOGICAL DIFFERENTIATION OF CONNECTIVE TISSUE

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Plates 17 to 19

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Recent investigations have shown (1–3) that the epithelium lining the kidney pelvis, ureter and urinary bladder is capable of directly inciting osteogenesis in certain connective tissue areas in the dog, guinea pig and rat. This can be demonstrated most easily by removing it from its normal environment by transplantation. Provided the transplanted epithelium survives, it proliferates to form a cyst containing brown cloudy fluid, lined with epithelium and encapsulated by fibroblasts. In the parietal connective tissues, for example those of the abdominal wall, extremities, neck and others, the fibroblasts surrounding a portion of the cyst readily and always undergo osteoblastic transformation and true bone is formed, containing fibrous and later blood-forming marrow. Other connective tissues such as those surrounding the epithelium in the urinary tract, the fibroblasts *within* the kidney cortex, liver and spleen, do not react to this epithelial stimulus, although the other conditions remain the same. Thus the evidence suggests that the mechanism preventing the formation of bone normally in the urinary tract lies in the presence of connective tissue functionally variant from the fibroblasts of the more parietal regions.

In the present communication, evidence will be presented to demonstrate that a similar osteogenic function is inherent in the epithelium of the gall bladder. During the course of the experiments, calculi composed chiefly of calcium carbonate were frequently observed and this phenomenon will also be briefly discussed, as will be further evidence that physiological variations exist in connective tissues, which appear morphologically similar.

LITERATURE

The Gall Bladder and Bone Formation.—The gall bladder has been transplanted in three previous experiments. Nakamoto (4), investigating carcinoma of the gall bladder, transplanted this organ to the abdominal wall in 18 guinea pigs and observed the formation of bone and cartilage within the transplanted organ; this author presented his results in brief form (one sentence) and made no mention of a causal histogenetic relationship. In the transplantation experiments of Stater (5) and Bauer and Hakki (6) bone was not observed.

A spontaneous occurrence in man of a process closely analogous to the present experimental findings was observed by Micseh (7) who reported a necropsy where carcinoma of the gall bladder was found with metastases to the liver, lung and lymph glands. Both the original tumor and the metastases contained true bone and hematopoietic bone marrow. On microscopic examination the tumor presented a papillary pattern in great part and the heterotopic bone was situated in connective tissue closely adjacent to the epithelial cells; in the tumor wherever bone was present, neoplastic epithelial cells were in intimate relationship to it.

Bone in the human gall bladder in close association with calculi as described by Phemister, Rewbridge and Rudisill (8) is probably not related to the phenomenon under present consideration.

The Gall Bladder and Calcium Carbonate Calculi.—The occurrence of these calculi in man has been considered by Phemister (8, 9, Lit.) who has constructed a clinical syndrome associated with calcium carbonate deposition in the gall bladder. Askanazy (10) has discussed the finding of $CaCO_3$ microliths at necropsy.

These stones have been observed experimentally by several investigators. Carmichael (11) introduced sterile and infected foreign bodies into the gall bladder of dogs and rabbits and found that calcium carbonate was deposited on the foreign surface. Rous, McMaster and Drury (12) found calcium carbonate in the proximal portion of the tubing used by Rous and McMaster in their device for collecting bile under sterile conditions. Walsh and Ivy (13) found calculi in the gall bladder of 3 dogs with occluded cystic duct. Phemister, Day and Hastings (9) described calcium carbonate calculi in rabbits and a dog following cystic duct ligation and injection of streptococci into the gall bladder.

Methods

Dogs and guinea pigs were used. The technique was that of transplantation. The animals were operated upon under ether, with asepsis, in 3 groups. In Group 1 the dome of the gall bladder was excised and replaced by a circular, free patch of connective tissue covering the rectus abdominis muscle, about 2.5 cm. in diameter; the repair was made by continuous suture with one layer of fine silk. Group 2 was identical to Group 1 except that the patch consisted of connective tissue, muscle and peritoneum obtained from the dome of the urinary bladder by denuding its mucosa. It was sutured in place so that the serosa faced the peritoneal cavity. In Group 3, the gall bladder was excised, opened and sutured to the fascia of the anterior abdominal wall and to the wall of the stomach. At the termination of the experiment x-ray photographs of the specimens were taken, after which serial sections of the material were made for histological examination.

RESULTS

Group 1. Transplantation of Abdominal Connective Tissue to Gall Bladder.—Four experiments were done and the dogs were killed at 46, 50 and 56 days. At necropsy in every case the transplant was found surrounded by omental adhesions, markedly shrunken, very hard to the touch and showing an area of calcium density in the x-ray photograph. Microscopic examination showed that the cylindrical epithelium had grown over the patch in a single layer of cells much less papillary than the original lining of the gall bladder but otherwise identical with it. This difference together with the presence of the sutures and the absence of smooth muscle clearly demarcated the transplant. In each instance there was found a thin plaque of bone with marrow spaces filled with connective tissue, confined strictly to the transplant. A narrow zone of fibroblasts, two to twenty cells in depth, separated the epithelium from the bone. The surface of the bone was covered with a palisade of osteoblasts and a few osteoclasts.

The possible influence of bile on this process was excluded by emptying the gall bladder and ligating the cystic duct at the time of transplant in 3 other dogs. In 2 dogs epithelium grew across the patch and bone was found in the subepithelial layers of the transplant, identical with that found in bile-containing gall bladders. In the third dog, due to infection, a pyogenic membrane formed on the surface of the transplant and bone was not found in this experiment. In each instance the gall bladder was found filled with mucus, in one instance thin and limpid, and in the others thick, tenacious and inspissated. In this mucus in all of the dogs, and embedded in the pyogenic membrane of the infected transplant in one, microliths were found and chemical examination of the larger of these showed them to be composed chiefly (91.4 per cent) of $CaCO_3$ with no trace of inorganic phosphorus. The largest stone was 3 x 2 x 1 mm. but the great majority were microscopic in size, 10–50μ in diameter. Some of these stones took a purple stain with hematoxylin, while others were colorless. Most of the stones were spherical and laminated in shape, but there also occurred a wide variety of configurations, ellipsoid, budding forms, etc.

Group 2. Transplantation of Urinary Bladder Connective Tissue and Muscle to Gall Bladder.—This procedure was carried out in 3 dogs and necropsy was made 36 days later. The gall bladders were not unduly distended, contained 8–15 cc. of bile. In each case there were adhesions about the gall bladder, and omentum was attached to the fundus. X-ray photographs in contrast to the previous cases failed to show bone. On section the surface of the patch was glistening; in one instance the suture material had partially extruded into the lumen at one point and the epithelium here was edematous.

TABLE I

Results in Group 3. Transplantation of Gall Bladder to Abdominal Wall in Guinea Pigs and Dogs

Guinea pigs	Dogs
13 days: Osteoid tissue	7 days: No bone
14 days: True bone present	16 days: No bone
15 days: No bone—infection	18 days: No bone
15 days: Osteoid tissue	29 days: No bone
17 days: True bone present	41 days: Bone present
17 days: True bone present	46 days: Bone present
19 days: True bone present	48 days: No bone
19 days: No bone	
21 days: Bone present	
23 days: Bone present	
23 days: Bone present	
27 days: Bone present	
34 days: Bone present	

Microscopic examination showed that cylindrical epithelial cells of the gall bladder had grown over the patch. There was a marked tendency of the epithelium to grow down along the silk between the patch and the gall bladder wall and in several places it reached the serosa. A partial atrophy of the smooth muscle of the transplant occurred, but numerous compact bundles of these cells could be identified easily. The epithelium covering the transplant rested on connective tissue. There was no formation of bone at any place.

Group 3. Transplantation of Gall Bladder Epithelium to Connective

the organism, manifesting itself in potency or impotence to form bone. Further data on physiological differences of fibroblasts have been obtained in other ways by Parker (14–16) and by the one of the present authors (2, 17). It is of interest that carcinomatous gall bladder epithelium has once been observed (7) to cause bone to form from the connective tissue of the gall bladder.

It is our concept derived from the evidence obtained in these experiments that this bone is not an unusual, bizarre response to the adjacent, geographically abnormal epithelium, but that it is a normal and usual reaction of certain connective tissues. Thus the evidence shows that it is the subepithelial connective tissue which fails to respond to the osteogenic stimulus of the overlying epithelium and thus prevents the formation of an osseous layer in the gall bladder under normal circumstances.

The total absence or the small size of the bone fragments in Group 3 must be considered. In each case the fibromuscular mechanism of the gall bladder was not dissociated from the epithelium. It is probable that growth of these transplanted fibroblasts, which are incapable of forming bone, occurred, sequestering the growing epithelium in large part or completely from the fibroblasts capable of forming bone.

The osseous transformation of the connective tissue develops after its cells have swollen and become basophilic to hematoxylin so that they correspond to osteoblasts. Osteoblasts are not present in these cell areas of the abdominal wall, and this observation is evidence that the osteoblast is derived by metaplasia of certain connective tissue cells.

The formation of the calcium carbonate microliths in the gall bladder mucus is related, as Phemister has shown, to the cystic duct occlusion. It is apparent that an abnormal surface such as the cannula system of Rous, the glass beads of Carmichael and the connective tissue patches as here, facilitates deposition and growth of the calculi.

SUMMARY

Evidence is presented that the proliferating gall bladder epithelium in the dog and guinea pig is capable of stimulating bone formation in certain connective tissues such as the abdominal wall. Other connective tissue areas such as the subepithelial connective tissue of the gall bladder and urinary bladder do not share in this tissue reaction and

resist the bone stimulus of the epithelium. The formation of bone in these circumstances is thus biphasic.

A difference between connective tissues morphologically identical can be proven physiologically, by their response to the osteogenic stimulus of appropriate epithelia.

Calcium carbonate microliths occurred in the mucus of the occluded gall bladder in which there was transplanted connective tissue forming part of the wall.

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EXPLANATION OF PLATES

The stain used in preparing the photomicrographs was hematoxylin and eosin.

PLATE 17

FIG. 1. Transplantation of connective tissue of the abdominal wall to the fundus of the gall bladder at 46 days. The epithelial surface is above, and the serosa (*S*) below. A thin layer of bone confined to the transplant is seen beneath epithelium. The bone has been decalcified. × 15.

FIG. 2. A higher magnification of a part of the epithelium and bone (*B*) of Fig. 1. × 185.

FIG. 3. Somewhat tangential section of abdominal wall transplant to fundus of gall bladder with occluded cystic duct, showing bone (*B*) in close association with the gall bladder epithelium. The bone has not been decalcified. × 70.

PLATE 18

FIG. 4. Transplantation of connective tissue and muscle of the urinary bladder to the dome of the gall bladder at 36 days. One-half of the transplant is shown with the serosal surface (*S*) below and the epithelium above. The normal gall bladder wall is at the left and between this and the transplant there has been an invagination of epithelium along the sutures. Smooth muscle bundles of the bladder wall may be seen. The epithelium has grown across the fibroblasts of the transplant but bone has not formed. × 15.

FIG. 5. Transplantation of gall bladder epithelium to abdominal wall of guinea pig at 17 days. Vesicles lined with gall bladder epithelium are seen with closely adjacent islands of partly calcified bone (*B*). × 125.

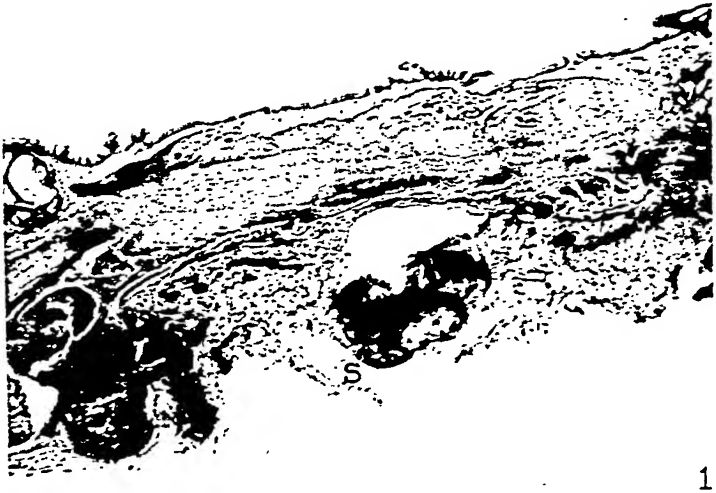
FIG. 6. Transplantation of gall bladder epithelium to abdominal wall in the dog at 42 days. Calcified bone is seen separated from the epithelium by a thin layer of fibroblasts. × 250.

PLATE 19

FIG. 7. Microliths composed chiefly of calcium carbonate embedded in granulation tissue of a fascial transplant to the gall bladder for 40 days. × 300.

FIG. 8. Same as Fig. 7, showing various shapes of the microliths. × 185.

FIG. 9. Same as Fig. 7. × 360.



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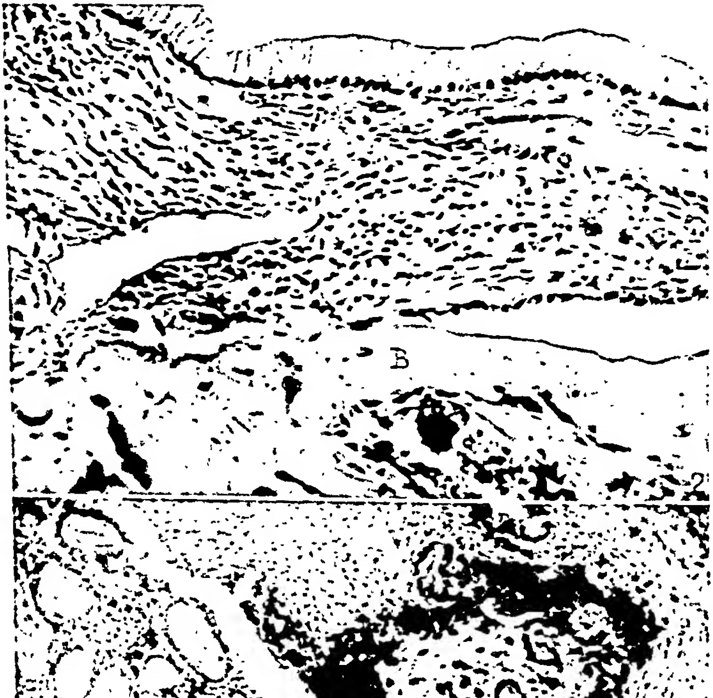

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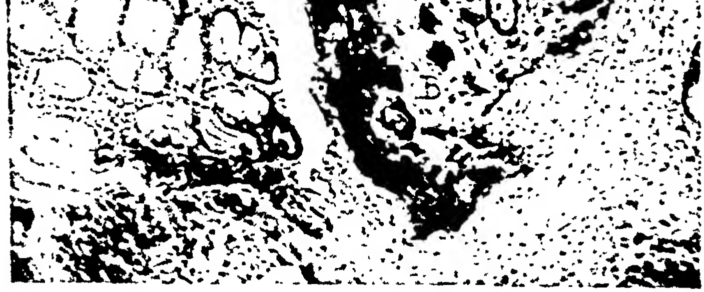

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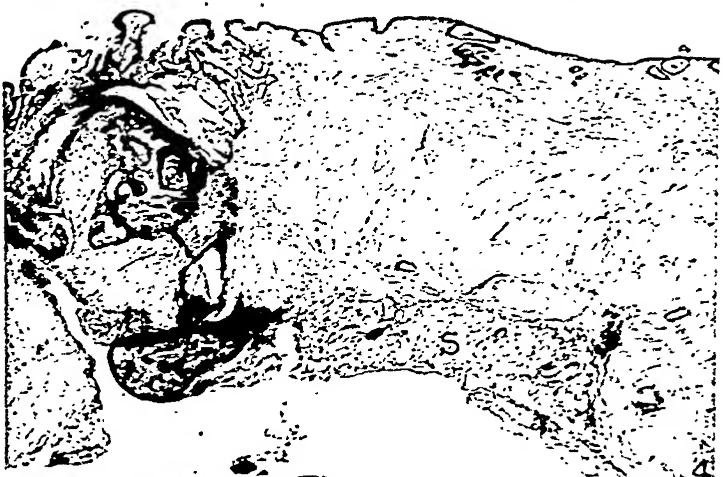
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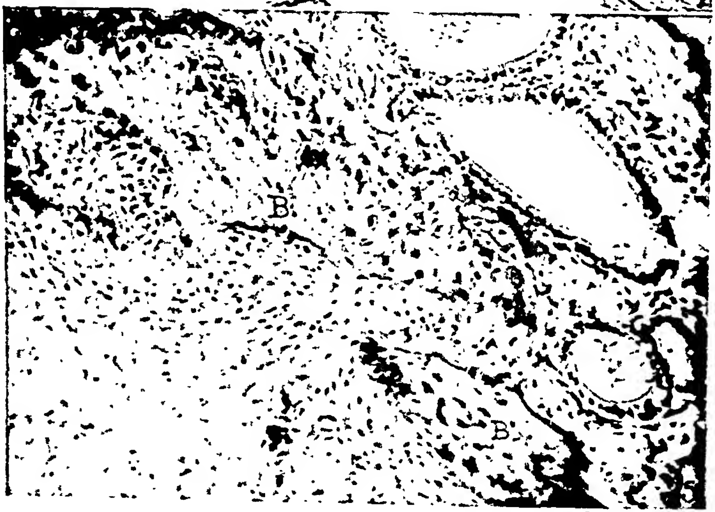
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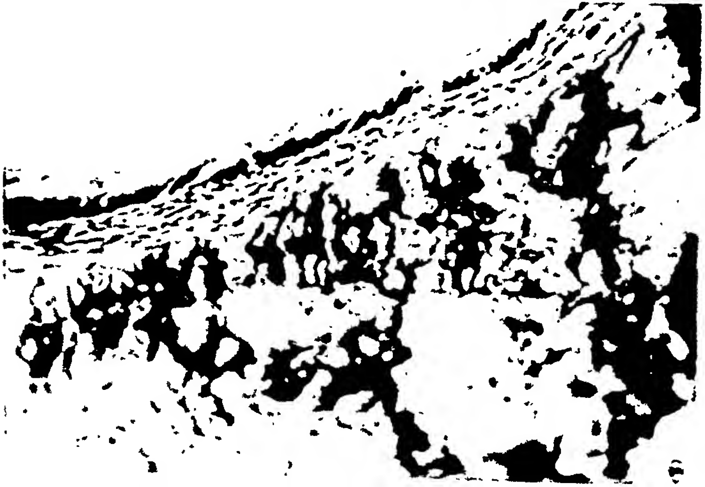

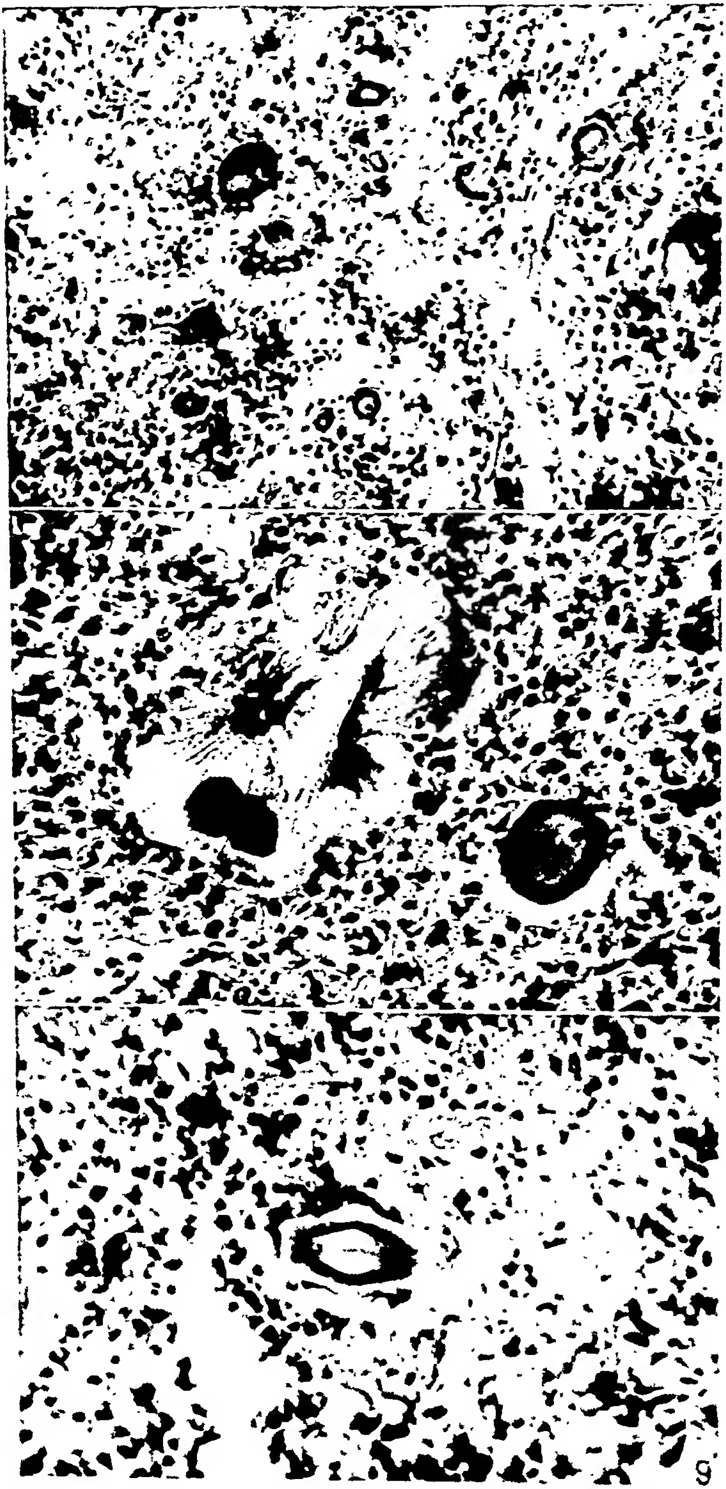

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THE RACES THAT CONSTITUTE THE GROUP OF COMMON FIBROBLASTS

III. Differences Determined by Origin of Explant and Age of Donor

By RAYMOND C. PARKER, Ph.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, June 6, 1933)

The purpose of the experiments described in this article was fourfold: first, to study the extent to which the functional differences that distinguish the various races of fibroblasts may be determined by the particular part of the organism from which they are derived; second, to study the extent to which these differences may be determined by the age of the individual from which they are obtained; third, to ascertain whether or not the differences are permanent; and fourth, to discover, if possible, the factors that are responsible for them.

Functional Differences Determined by the Origin of the Explant

Five series of strains have been studied, each series comprising eight to twenty races of fibroblasts that were isolated simultaneously from different parts of a single chick embryo and cultivated for as long as 10 successive weeks (ten passages) in a medium consisting of chick plasma and chick embryonic tissue juice diluted with Tyrode solution. Of the five series, four were derived from 16 day chick embryos and one from a 17 day embryo. The strains were obtained from the following tissues and organs: bone, cartilage, liver, lung, mesonephros, metanephros, ovary, pancreas, salivary gland, spleen, testis, thyroid, musculature of the heart, breast, esophagus, crop, proventriculus, gizzard and small intestine, skeletal musculature of the lower limb, and wall of the aorta. From the moment of their isolation, the various strains comprising each series were cultivated under conditions as nearly identical as it was possible to make them. The techniques employed were the usual ones involving the flask procedures. After a few passages, the cell population was rendered uniform by selection of only the marginal areas of outgrowth at the time of transfer.

Aside from making a comparative study of the rate of growth of the various cell races, and of their ability to digest the fibrin of the coagulum, tests were carried out from time to time to determine the relative changes that took place in the

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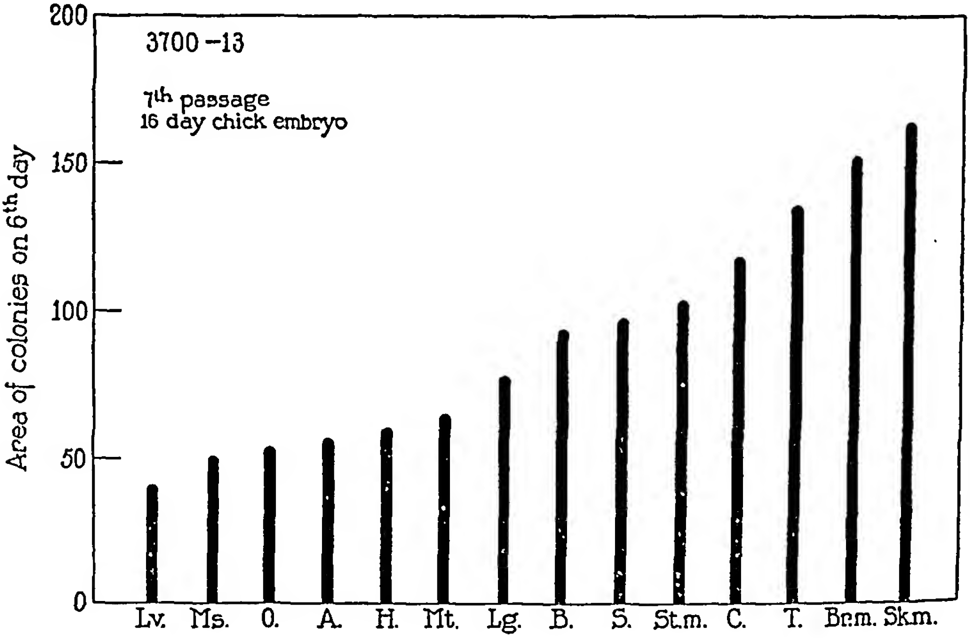
Aside from making a comparative study of the rate of growth of the various cell races, and of their ability to digest the fibrin of the coagulum, tests were carried out from time to time to determine the relative changes that took place in the acidity of the medium in which they were cultivated. For this purpose, a dilute solution of phenol red (0.005 per cent) was added to the medium, after which the hydrogen ion concentration was adjusted to 7.6 by introducing into the flask a gas mixture comprised of 3 per cent CO_2, 21 per cent O_2, and 76 per cent N (at atmospheric pressure). The changes produced in the various cultures were then read at 24 or 48 hour intervals by comparing them with a standard series of flasks of known pH values.

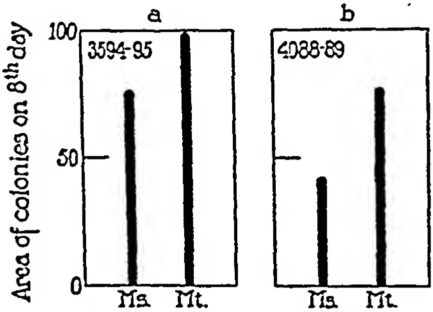


TEXT-FIG. 1. Diagram representing the rate of multiplication of fourteen strains of fibroblasts isolated from the liver (*Lv.*), mesonephros (*Ms.*), ovary (*O.*), aorta (*A.*), heart (*H.*), metanephros (*Mt.*), lung (*Lg.*), bone (*B.*), spleen (*S.*), stomach musculature (*St. m.*), cartilage (*C.*), thyroid (*T.*), breast musculature (*Br. m.*), and skeletal musculature (*Sk. m.*), of a 16 day chick embryo, and cultivated in a mixture of plasma and embryonic tissue juice; from planimetric measurements of the areas attained by the colonies on the 6th day of the seventh passage.

Other experiments were designed to test the ability of the various strains to survive and grow in a medium of relatively high acidity. Here, the cultures were subjected to a gas mixture composed of 20 per cent CO_2, 21 per cent O_2, and 59 per cent N, this combination having been found to produce a pH of 6.5.

The results of the experiments showed that the various races, according to their origin, differed from one another in their rate of multiplication in a given medium, in the relative changes that took place in the acidity of that medium, in the relative degree of acidity they were able to withstand, and also in their ability to digest the fibrin of the coagulum.

The rate of multiplication of fourteen strains comprising one particular series is represented diagrammatically in Text-fig. 1. These strains were isolated from aorta, bone, breast musculature, cartilage, heart, liver, lung, mesonephros, metanephros, ovary, skeletal musculature, spleen, stomach musculature, and thyroid of a 16 day embryo, and were cultivated for 72 days (eleven passages) under the same environmental conditions. The major differences in the rate of proliferation manifested by the various strains on a common medium were maintained from passage to passage indefinitely. As can be seen from Text-fig. 1, fibroblasts derived from thel iver displayed the lowest growth energy of any of the series, whereas those from skeletal muscle showed the highest. The strains that possessed a high growth energy, for example fibroblasts from skeletal muscle, breast muscle, etc., liberated a relatively large amount of acid into the medium and were able to withstand a medium of high acidity. These properties were not necessarily accompanied, however, by a marked ability to digest fibrin. Thus, while fibroblasts derived from the lung and spleen,

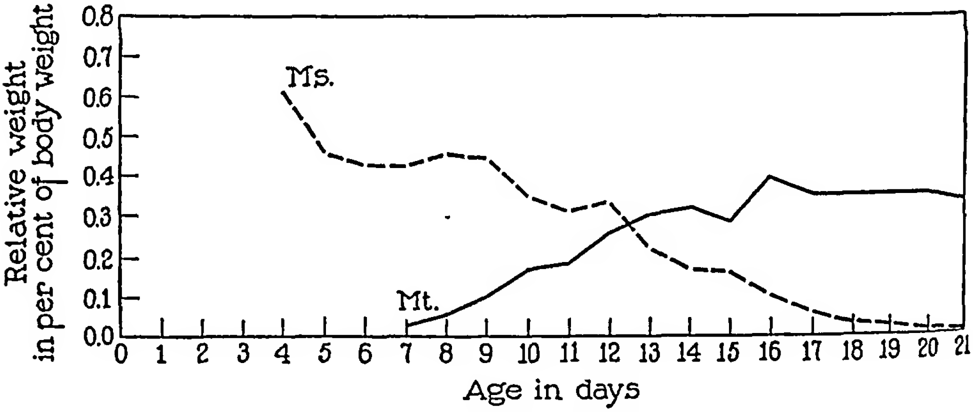


Text-Fig. 2. (*a*) Diagram representing the rate of multiplication of strains of fibroblasts isolated from the mesonephros (*Ms.*) and metanephros (*Mt.*) of a 16 day chick embryo, and cultivated in plasma and embryonic tissue juice; from planimetric measurements of the areas attained by the colonies on the 8th day of the fifth passage.

(*b*) Another experiment made on similar strains from an embryo of the same age.

and from the various regions of the digestive tract, multiplied, as a general rule, at a slower rate and produced less acid, they rapidly digested the fibrin of the coagulum. This was also true of fibroblasts from the thyroid, although in this case the colonies grew in an extremely thin layer, much thinner than has ever been observed for other races of fibroblasts.

Of particular interest, however, were the strains derived from the mesonephros and metanephros, respectively. It will be noted that the cells from the metanephros multiplied more rapidly than those from the mesonephros (Text-figs. 1 and 2*a*). Some years ago, Schmalhausen[1] weighed and measured a great number of the component structures of developing chick embryos. For presentation at this time, a graph has been constructed from a table in which he showed the relative increase in weight of various embryonic organs throughout the incubation period (Text-fig. 3). On the 16th day, when the above mentioned strains were isolated, it will be seen from Schmalhausen's data that the metanephros, or permanent kidney, is undergoing rapid development. On that same day, the mesonephros, a more primitive structure, is disappearing. From the standpoint of the present investigation, it was most significant, therefore, that the cells derived from



TEXT-FIG. 3. Changes in the relative size of the mesonephros (*Ms.*) and metanephros (*Mt.*) during the embryonic life of the chick, expressed as percentages of the total weight of the body (constructed from Table 6, Schmalhausen[1]).

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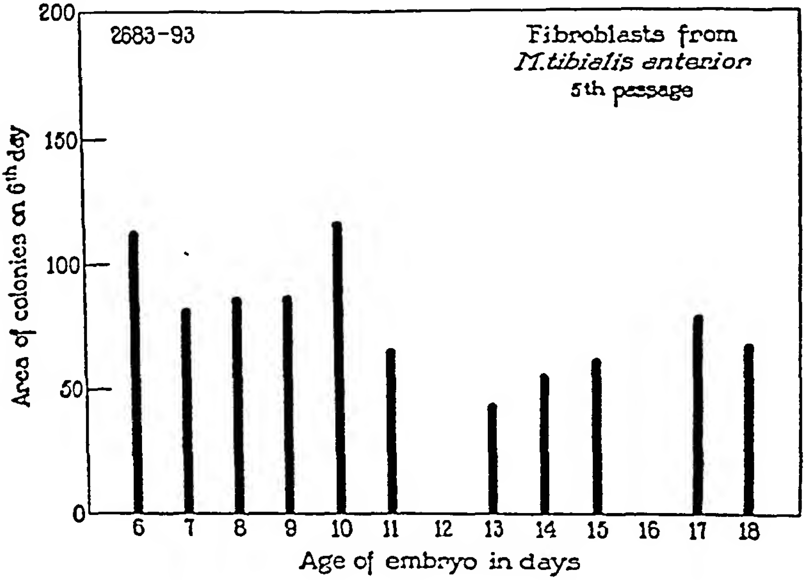
[1] Schmalhausen, J., *Arch. Entwcklngsmechn. Organ.*, 1927, 110, 33.

the developing metanephros proliferated more actively than those from the degenerating mesonephros. This experiment was later repeated with strains isolated from corresponding parts of another embryo of the same age and species. The results were comparable with those already obtained (Text-fig. 2 *b*).

Functional Differences Determined by the Age of the Donor

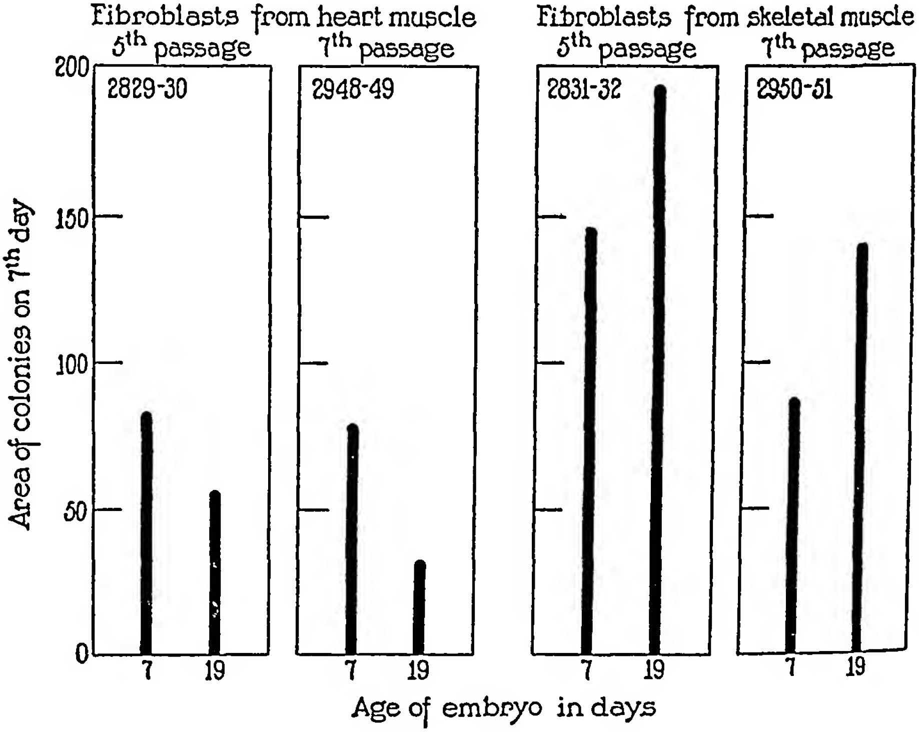
Two groups of experiments were made in order to study the extent to which the functional properties of fibroblasts are determined by the age of the embryo from which they are derived. The first group included five series, each series consisting of five to twelve strains isolated from the apex of the ventricle of embryos ranging from 6 to 20 days in age. The second group also included five series of five to twelve strains each, these having been isolated from the *M. tibialis anterior* of embryos of different ages. Comparative studies were made of the relative abilities of the various strains of each series to multiply in a medium consisting of plasma and embryonic tissue juice diluted with Tyrode solution. This



TEXT-FIG. 4. Diagram representing the rate of multiplication of eleven strains of fibroblasts isolated simultaneously from the *M. tibialis anterior* of chick embryos of eleven different ages, and cultivated in plasma and embryonic tissue juice; from planimetric measurements of the areas attained by the colonies on the 6th day of the fifth passage.

was done by recording their rate of proliferation for each subsequent passage over a period of weeks. As before, the strains comprising each experimental series were isolated at one time and were treated alike throughout the entire period of their cultivation.

The rate of multiplication of the fibroblasts differed according to the age of the embryo from which they were isolated. There was never, in all the series that were studied, a gradual increase or decrease in the rate of proliferation of strains isolated from corresponding parts of embryos of increasing ages (Text-fig. 4). It was frequently found, for example, that muscle fibroblasts from embryos around 13 days of age multiplied more slowly than those from either older or younger embryos. Also, there were often decided peaks in the proliferative



Text-Fig. 5. Diagrams representing the rate of multiplication of strains of fibroblasts isolated simultaneously from the heart and skeletal musculature of 7 and 19 day chick embryos, and cultivated in plasma and embryonic tissue juice; from planimetric measurements of the areas attained by the colonies on the 7th day of the fifth and seventh passages, respectively.

rate of strains that were isolated on or about the 10th and 17th days of embryonic life.

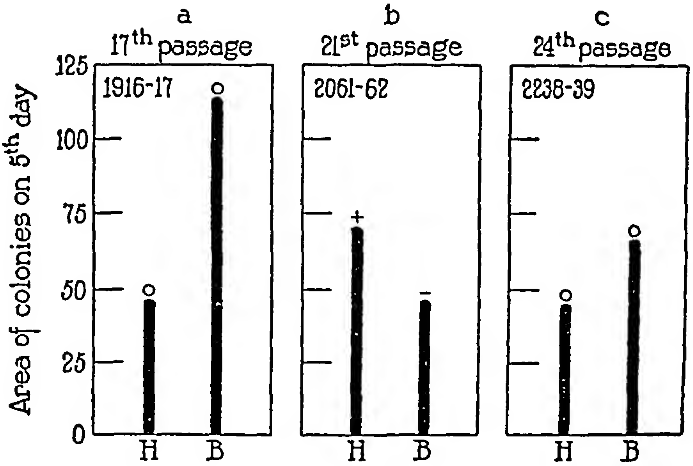
The series of strains from the developing heart behaved quite differently from those obtained from muscle. To illustrate this, reference is made to an experiment including cell races that were derived simultaneously from both the heart and skeletal muscle of 7 and 19 day embryos, respectively, and cultivated under the same environmental conditions from the moment of isolation (Text-fig. 5). Although the heart fibroblasts obtained from the younger embryo proved to be more active than those derived from the older, the effect of age was quite the reverse in the case of fibroblasts from the skeletal musculature.

It was also observed that, whereas the major differences found to exist between the various strains belonging to a given series were maintained from passage to passage indefinitely, it could not be predicted from one particular series how the strains of a second series would behave, even when the tissues were isolated from corresponding parts of embryos of the same age and species. As a matter of fact, it was never safe to assume that two cell strains would react identically to the same conditions, even when they were isolated simultaneously from closely adjacent parts of the same tissue or organ and cultivated together on the same medium. Furthermore, the two halves of a single culture could not be relied upon to display the same properties until the strain it represented had become homogeneous and composed solely of fibroblasts. In order to attain this condition, it was necessary to pass the cultures through several passages and to discard all but the outer margin of outgrowth.

The Ability of the Various Races to Retain Their Functional Characteristics Indefinitely

It has already been intimated that the functional differences distinguishing the various races of fibroblasts are permanent, that they persist indefinitely *in vitro*. Even after months of cultivation, strains that were isolated from different parts of the same embryo continued to manifest those properties that had characterized them in the beginning. Cell strains endowed with a high capacity for multiplication on a given medium continued to manifest that property. Strains possessing an unusual ability to digest fibrin continued to do so for passage after passage. This was equally true of differences dependent upon the age of the individual.

In an attempt to change the character of certain races by altering the nature of their environment, two groups of experiments have been made. The first group was designed to test the permanence of differences determined by the particular organ or tissue supplying the strains (Text-fig. 6); the second, to test the permanence of differences determined by the age of the embryo from which the strains were derived (Text-fig. 7).



TEXT-FIG. 6. Diagrams representing the rate of multiplication of strains of fibroblasts isolated simultaneously from heart and bone of a 13 day chick embryo, and afforded identical treatment from the beginning until the 97th day.

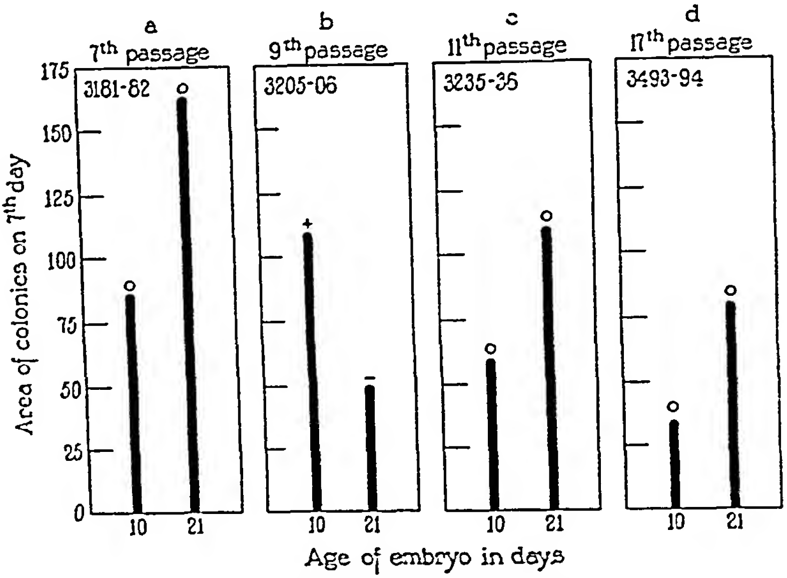
(*a*) The behavior of the strains for the final passage of this period of identical treatment (o).

(*b*) The rate of multiplication of the same strains for the last of four successive passages during which time the fibroblasts from heart were subjected to more than the usual amount of embryonic tissue juice (+), and the fibroblasts from bone to less than the usual amount (−).

(*c*) The rate of multiplication of the strains after the previous treatment had been discontinued, and both strains had again been cultivated in the same medium (o) for three successive passages.

The material used in connection with the first group of experiments consisted of two races of fibroblasts from a 13 day chick embryo, one race having been isolated from the ventricle of the heart, the other from the periosteum of bone.

Both strains were treated alike for 97 days (seventeen passages), during which time they were cultivated in a medium consisting of chick plasma and chick embryonic tissue juice diluted with Tyrode solution (Text-fig. 6 *a*). At the nineteenth passage, the two strains were transferred to media of different composition,



TEXT-FIG. 7. Diagrams representing the rate of multiplication of strains of fibroblasts isolated simultaneously from the *M. tibialis anterior* of 10 and 21 day chick embryos, and subjected to identical treatment from the beginning until the 38th day.

(*a*) The behavior of the strains for the final passage of this period of identical treatment (o).

(*b*) The rate of multiplication of the same strains for a 7 day period, during which time the fibroblasts from the 10 day embryo were treated with more than the usual amount of embryonic tissue juice (+), whereas those from the 21 day embryo received less than usual (−).

(*c*) The rate of multiplication of the strains after the previous treatment had been discontinued, and both strains had again been cultivated on the same medium (o) for two passages.

(*d*) The rate of multiplication of the strains after they had been cultivated on the same medium (o) for an additional period of six passages.

the heart fibroblasts being cultivated in a medium containing much more than the usual amount of embryonic tissue juice, while the fibroblasts from bone were placed in a medium in which the quantity of tissue juice was greatly diminished.

This treatment was continued for 26 days, through four passages (Text-fig. 6 *b*). At the end of that time, when the overfed heart fibroblasts were dividing at a much higher rate than the starved bone fibroblasts, the two strains were again placed under the same environmental conditions. Almost immediately, they reverted to their original rate of multiplication, and after three passages on a common medium they both displayed the same characteristics that had distinguished them in the beginning (Text-fig. 6 *c*).

The second group of experiments was carried out in much the same manner. Two races of fibroblasts were isolated simultaneously from skeletal muscle removed from the lower limbs of 10 and 21 day chick embryos. When cultivated under the same environmental conditions, the cell strain derived from the 21 day embryo showed a rate of multiplication that was consistently higher than that manifested by the strain from the younger embryo (Text-fig. 7 *a*) At the ninth passage, the 10 day strain was treated with more than the usual amount of embryonic tissue juice, the 21 day strain with less (Text-fig. 7 *b*). The result of this was similar to that obtained with the races from heart and bone. The rate of multiplication of the strain from the 10 day embryo, which had originally been much lower than that of the strain from the older embryo, now became the greater of the two. After this treatment had been discontinued, the two races were again placed in media of like composition. The original characteristics reappeared (Text-fig. 7 *c*), and continued to persist until the termination of the experiment, at which time the two types of fibroblasts had been cultivated *in vitro* for a total period of 109 days (Text-fig. 7 *d*).

DISCUSSION

The experiments have shown that the ability of the common connective tissue cells, or fibroblasts, to multiply in a given environment depends not only upon the nutritional quality of the latter, but also upon the inherent capacities of the cells themselves. Fibroblasts, as a group, comprise many cell races. Each race manifests certain specific functional properties when cultivated *in vitro*. These properties differ according to the age of the individual and also according to the particular organ or tissue from which the strain is derived. They are displayed in the relative abilities of the various races to live and reproduce in a given environment, to produce marked changes in the acidity of that environment, and to digest fibrin.

It has generally been assumed that the rate of multiplication of fibroblasts cultivated *in vitro* decreased in simple inverse ratio to the age of the individual from which they were isolated. While this may be true of fibroblasts isolated during postnatal development, the present experiments have shown that it does not hold for cell races obtained, from certain organs at least, during the embryonic period. Thus, for example, cell strains derived from a 21 day embryo may show, under the same environmental conditions, a rate of multiplication that is either higher or lower than that exhibited by strains isolated from corresponding parts of a 10 day embryo, depending upon their place of origin. As already indicated, it was impossible to demonstrate a gradual decrease, or even a gradual increase, in the rate of multiplication of fibroblasts obtained from corresponding parts of embryos of increasing ages.

In the beginning, the marked irregularities that were observed to occur in the relative growth rates of strains derived from embryos of gradually increasing ages could not be accounted for. At first, it was felt that they might have resulted from gross errors in technique. As the experiments progressed, however, it became evident that the major differences that were observed between the various strains of each series were maintained from passage to passage indefinitely. Not only were these differences real, they were also permanent. In this respect, they were comparable with those differences that had been found to exist between strains isolated from different parts of the same embryo.

Although it was now clear that the major irregularities observed in the various series did not result from the kind of treatment they received, it still seemed possible that, if the tissues were not derived from corresponding locations, small differences in origin might be sufficient to produce them. Accordingly, the original explants were selected with even greater precision in order to obtain the fragments from exactly the same location in each embryo. Despite this precaution, however, the irregularities continued to appear in each series that was studied. A strain of fibroblasts derived from a 10 day embryo showed a rate of multiplication that was greater than that of all other strains belonging to the same series, regardless of whether they were isolated from younger or older embryos. It became apparent, therefore, that these properties of the various strains were in some way related to conditions existing in the tissues of the organism prior to their isolation.

As already mentioned, Schmalhausen[1] weighed and measured a great number of the component structures of developing embryos in an attempt to gain precise information as to some of the time relations of development. He calculated the percentage growth rates and also the relation between the weights of the organs, expressed as percentages of the total weight of the body. The growth rate of each organ was found to depend upon its own age rather than the age of the entire organism. Also, while the majority of the organs studied made their appearance in the embryo at different times, their percentage growth rates showed definite changes throughout the course of embryonic development. Almost every organ showed, at some time during its development, a marked peak in its percentage growth rate, which peak was immediately followed by a gradual decline. Each of these changes resulted from a short period of rapid increase in the percentage growth rate of the organ, followed by a like period of decrease. The amount of this rise and the time of its occurrence differed in different cases, and was thus a characteristic of the organ itself. Even during the descent, however, the growth rate showed a similar series of abrupt changes, each expressing periods of increase followed by periods of decrease, and each being less pronounced than the one before. As a rule, all of these changes were synchronous for the various organs.

The findings of Schmalhausen,[1] together with the results of the present experiments, suggest that the rate of proliferation shown by the component cells of the various organs upon isolation depends solely upon whether the part removed from the organ in question was, at the moment just prior to isolation, going through a period of increase or one of decrease. If this is so, it would also depend upon whether the rate of increase, or decrease, at the moment was rapid or gradual. In other words, the results indicate that the physiological condition of the organized structures is reflected in the functional properties of their component elements. This contention has been amply sustained by the observations reported in connection with the cell strains obtained from the mesonephros and metanephros at the time when the latter was giving place to the former. Under the same environmental conditions, the fibroblasts derived from the developing metanephros multiplied at a faster rate than those from the degenerating mesonephros.

Some years ago, Cohn and Murray,[2] who were among the first to

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[2] Cohn, A. E., and Murray, H. A., Jr., *J. Exp. Med.*, 1925, 42, 275.

apply the *in vitro* techniques to the study of physiological ontogeny in the chick, measured the latent period and the growth index of fragments of heart that were isolated from 4 to 18 day old embryos and placed for incubation in coagulated chick plasma. The latent period, which was defined as the time that intervened between the incubation of a culture and the first appearance of cells protruding from the peripheral margin, was found to increase with age. The growth index was estimated from the area of the original fragment and the area of outgrowth after 24 and 48 hours. Unlike the latent period, it showed a marked decrease with age. The conclusion was reached that differences due to age signified a habit of slow growth imposed upon the cells by definite changes in the organized environment of the embryo. After 36 hours or so, this habit was lost and the tissues grew at a rate determined solely by their environment.

In so far as the latent period was concerned, Cohn and Murray[2] went no further than to show that it was related in some way to the age of the tissue. Even today, nothing more can be said. At any rate, there is no legitimate reason for assuming that there is a direct correlation between the latent period of outward cell migration and the specific properties of the cells directly concerned. Marked changes are known to take place in the organs of the individual during development. It usually happens, for example, that there is a gradual increase in the density of the connective tissue proper and in the amount of intercellular substance it contains. This being true, the cells of older organs would, of necessity, require a longer time to invade the medium than those of younger structures that are less densely populated and of looser texture.

The techniques in use at the time of the earlier experiments were extremely primitive. Furthermore, it was not unusual to limit observations of a functional nature to the behavior of tissues freshly extirpated from the organism, thereby eliminating the possibility of obtaining pure strains. In the case of Cohn and Murray's[2] work, this procedure was considered adequate at the time, inasmuch as it was assumed by them that they were dealing mainly with fibroblasts, and that all fibroblasts in the body were alike. It had not yet been fully realized that a fragment of tissue freshly explanted from the organism contains a great variety of cell types, and that marked differences may sometimes exist between fragments coming from different parts, even when these parts are closely adjacent.

Aside from the information that has been obtained concerning the different races that constitute the group of common fibroblasts, the present experiments have indicated, therefore, the necessity of exercising the greatest possible care in selecting material for comparative physiological studies on cells *in vitro*. They show that pure strains and controlled environmental conditions are fundamental prerequisites for each and every attempt that is made to investigate the functional properties of cells, and how these cells may affect, or be affected by, the medium in which they are cultivated.

SUMMARY AND CONCLUSIONS

1. Races of fibroblasts that are functionally distinct have been isolated from the various tissues and organs of a single chick embryo.
2. Functionally distinct races of fibroblasts have also been isolated from corresponding parts of embryos of different ages.
3. Under the conditions of the experiments, and for the particular races of fibroblasts that have been studied, it has not been possible to demonstrate a gradual decrease, or a gradual increase, in the rate of multiplication of fibroblasts obtained from corresponding parts of embryos of gradually increasing ages.
4. Experiments made on strains of fibroblasts derived from the mesonephros and metanephros of a 16 day chick embryo have indicated that the rate of multiplication of these cells in a given medium reflects the physiological state, at the moment of isolation, of the particular part of the embryo from which they are obtained.
5. The rate of multiplication of a given race of fibroblasts in a particular medium does not serve, necessarily, as an index of the age of the individual from which the race is derived.
6. The functional differences that distinguish the various races of fibroblasts are permanent; they are retained by the cells from passage to passage indefinitely, despite such attempts as have thus far been made to change them.

STUDIES ON PSEUDORABIES (INFECTIOUS BULBAR PARALYSIS, MAD ITCH)

I. HISTOLOGY OF THE DISEASE, WITH A NOTE ON THE SYMPTOMATOLOGY

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PLATES 20 TO 22

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Pseudorabies has now been reported as occurring in Hungary (Aujeszky, 1902, and many others), Brazil (Carini and Maciel, 1912), United States (Shope, 1931, 1932), Denmark (Bang, 1932) and Holland (Burggraaf and Lourens, 1932). Although in this disease nervous symptoms are prominent, usually no lesions other than vascular congestion have been found in the nervous system. Følger (1932) found hemorrhages and less often perivascular infiltration in cattle, but not in cats or rabbits. Among others, Bertarelli and Melli (1913), Sangiorgi (1914) and Følger have failed to detect cellular inclusions. In rabbits and guinea pigs, the extraordinarily rapid course of the malady, measured in hours, presumably allows little time for the development of gross pathological changes. Nevertheless characteristic lesions, identical whether the Aujeszky virus or Shope's Iowa strain ("mad itch") is employed, are usually present. In the following pages a description of these lesions is given.

Technique

The source of virus consisted of the supernatant fluid from a 10 per cent suspension of brain tissue of rabbits succumbing to intracerebral inoculation; no growth of visible bacteria occurred on culture. The experimental animals were killed when moribund, or obtained immediately after death, and their nervous systems subjected to full examination by neuropathological methods.

Lesions in the Rabbit

After Intradermal, Subcutaneous and Intramuscular Inoculation.—Locally, after 12 hours, there was a lively inflammatory response indistinguishable from that following the introduction of normal brain emulsion similarly prepared; polymorphonuclear leucocytes, lymphocytes and a few macrophages were present, but no cellular inclusions were seen. From 16 hours onward changes became progressively more marked and greatly surpassed those due to normal brain, which gradually subsided. Occasional inclusions (see below) were present in connective tissue and capillary endothelial nuclei. At 24 to 30 hours cellular infiltration was more intense; large numbers of polymorphonuclear leucocytes, many karyorrhectic or ingested by macrophages, marked the centre of the lesion, while at the periphery lymphocytes and eosinophils were numerous. Muscle fibres stained poorly, with diminished striation. Inclusions were rather more numerous. From 40 hours onwards extensive necrosis of muscle or connective tissue was evident; even where a minimal amount of virus had been introduced by scarification, a tiny necrotic focus could be found in the subepithelial tissues. Necrosis was thus not dependent upon the trauma of biting and scratching which followed later. Inclusions, though not numerous, could now be detected also in lymphocytes, macrophages, epidermal cells bordering a scratch, and occasionally in sarcolemmal nuclei. The local lymph glands were acutely inflamed.

The finest nerve twigs and nerve endings participated, of course, in this local inflammation. By the 40th hour definite signs of inflammation were visible in the nerve leaving the inoculated area. Lymphocytes, a few polymorphonuclear leucocytes and occasional macrophages occurred in the connective tissue sheath, around small vessels and in small foci between the nerve fibres. Careful search revealed scanty inclusions in the nuclei of the sheath of Schwann. During the period of the developed disease similar changes were sometimes found in the upper part of the sciatic nerve (after inoculation into the calf); lower down, though variable in degree, they were now much more marked, and their development was possibly assisted by trauma and secondary infection.

During the incubation period no changes were discernible in the spinal ganglia and segments of the spinal cord corresponding to the site of inoculation. About the time when itching commenced, *i.e.* 1 to 2 hours after virus could first be detected in the ganglia, early lesions affecting a few cells were apparent. In animals surviving a further 10 or 12 hours, nearly every cell of the corresponding ganglia was affected (Fig. 2) and lesions had appeared on the opposite side. The sequence of events was best seen in sections stained with phloxin-methylene blue after sublimate-formol or Zenker-formol fixation. In brief, oxychromatic degeneration of the nuclei resulted in the formation of intranuclear inclusions of the general type of those in herpes, yellow fever, etc.; cytoplasmic degeneration and necrosis followed.

The earliest changes, in the form of definite increase of oxyphilic material grouped in deep red, granular aggregates around the nucleolus, were met with in ganglion cells exhibiting normal contour of nucleus and body and well preserved nucleolus and Nissl substance; these masses were connected by fine threads with similar smaller masses near the nuclear membrane, but most of the nucleus was empty (Fig. 1 *B*). Rather later the nucleolus disappeared (Figs. 1 *C*, and 3). In two cases at an early stage considerable enlargement occurred in some nucleoli, chiefly of the acidophilic constituent; this appearance was not constant. The nucleus might now become completely filled with pale pink, finely particulate material surrounding small, deep red aggregates and sometimes fragments of the basophilic part of the nucleolus; most of the scanty basophilic chromatin was, however, marginated on the nuclear membrane (Fig. 1 *D* and *E*, Fig. 3). In a few cells the included material was less definitely acidophilic and assumed a mauve tint; sometimes, also, it appeared homogeneous rather than particulate. In the rabbit the former variation occurred only in the spinal ganglia.[1] Up to this point the Nissl substance, Golgi net, neurofibrils and mitochondria remained intact, but as the nucleus became completely filled with acidophilic substance they underwent disintegration; the mitochondria persisted in a swollen condition, and the fragmenting Golgi apparatus was visible for some time after the Nissl substance had disappeared. Progressive shrinkage of the nucleus, wrinkling and fading of the nuclear membrane and increasing acidophilia of the cytoplasm now heralded complete destruction of the cell (Fig. 1 *F*). Meanwhile rather similar, but often more obviously granular masses appeared in the nuclei of the capsule cells; here nuclear enlargement and margination of basophilic chromatin were often more pronounced, and the inclusion was always clearly acidophilic (Fig. 1 *E*, *F*, *G*, Fig. 3). With exceptionally long survival of the animal (over 12 hours after itching began), evidence of cellular reaction in the form of polymorphonuclear infiltration with commencing lysis of the necrotic nerve cells might be present; a large proportion of the polymorphonuclear leucocytes were karyorrhectic. Occasionally, too, neuronophagia by proliferated capsule cells was seen (Fig. 1 *G*).

In the posterior horn of the cord similar lesions appeared perhaps slightly later; nerve cells, glial cells of the grey matter and of the posterior root entry zone all were affected. At a very late stage scanty infiltration with polymorphonuclear leucocytes might be seen. In the anterior horns, glial cells showed inclusions much less commonly; only once were inclusions seen in anterior horn nerve cells.

In the posterior nerve roots nuclear changes in the Schwann cells were more common than in the proximal part of the nerve supplying the site of inoculation.

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[1] Saguchi (1930) has recently pointed out that the chromophilic state of neurons is characterized by overproduction of "nucleonephelium." Normally acidophilic, this substance may in the chromophilic state of the cell become basophilic and, dissolved in the nuclear juice, impart diffuse basophilia to the whole nucleus. This phenomenon is more common in the guinea pig, in which animal, as will be seen later, a much greater proportion of affected nuclei, even when fixed in Zenker's fluid, fail to show clearly acidophilic inclusions.

Although virus was usually present in small amount, no lesions were apparent in the cord and spinal ganglia at higher and lower levels, or in the brain stem, cerebellum or cerebrum. Their absence from these regions possibly accounts for previous failures to detect lesions in the central nervous system.

Intracerebral Inoculation.—At the site of inoculation, hemorrhage with some polymorphonuclear and eosinophil exudation but no massive necrosis was present; with an incubation period of about 28 hours, microglial reaction was very slight. The most prominent lesion was marked infiltration of the overlying meninges with polymorphonuclear leucocytes, lymphocytes, eosinophils and a few macrophages; many of the infiltrating cells were fragmented. With subdural inoculation (or leakage of inoculum into the meninges) meningitis was more intense and widespread; fibrinous exudation and necrosis occurred, the latter especially if the incubation period was slightly prolonged. In these cases inflammatory cells might extend along the perivascular spaces as deeply as the first or second layer of cortical nerve cells. A few polymorphonuclear leucocytes might overflow into the superficial nervous tissue.

Characteristic nuclear changes were found in almost every mesothelial cell of the pia-arachnoid, and in a large proportion of the subpial glial cells; in both types of cell they occurred beyond the limits of meningeal infiltration, and in many regions were the only abnormalities present (Figs. 1 *H*, and 4). They were seen less often in lymphocytes and macrophages of the meningeal exudate, in capillary endothelium, in adventitial cells of veins and arteries and in ependymal cells. Involvement of nerve cells and deeper glial cells depended largely on their proximity to a surface. Early nuclear inclusions, usually without cytoplasmic alterations, were not infrequent in superficial cortical neurons, in those of the fascia dentata and in Purkinje cells of the cerebellum (Fig. 5), while very superficial nuclei (*e.g.* the ganglion basale opticum) might show a majority of cells involved; adjacent glial cells suffered similar change. Deeply placed nerve cells, except those immediately adjacent to the site of inoculation, were mostly perfectly preserved, though some of the brain stem nuclei exhibited occasional lesions. Finally, inclusions were sometimes seen in the epithelioid cells of distant nodules due to infection with *Encephalitozoon cuniculi.*

Early inclusions were occasionally present in cells of the Gasserian ganglion. No definite changes were noted in the spinal cord.

Lesions in Other Organs.—Pathological changes commonly seen outside the nervous system were petechial hemorrhages in the thymus, and areas of intense congestion, hemorrhage and edema in the lungs. Edema of the lungs might also occur without marked congestion, or be of much more extensive distribution than the sanguineous areas. The hemorrhagic areas varied from spots a millimetre or two in diameter to areas occupying the whole of a lobe or even of several lobes; they were frequently present in animals dying of the disease, though probably not so frequently as in Shope's earlier cases.

When considered in relation to the virus content of the organ, the microscopical changes in the lungs were somewhat confusing. In the first place the lung might appear normal, and yet on occasion contained virus.[2] Secondly, in the absence of any macroscopic abnormality, the alveolar walls over large or small areas might show a marked excess of polymorphonuclear leucocytes; occasionally in association with this there occurred a few foci in which the alveolar epithelium had proliferated and undergone desquamation to mingle with extravasated polymorphonuclear leucocytes, often karyorrhectic. In one such case virus was not detected in the organ; in a second (infected with Aujeszky virus), not only was virus present, but occasional nuclear inclusions were found in the epithelial cells. (The latter observation was not repeated in a large series of lungs.) Thirdly, great congestion and alveolar hemorrhages, with or without serous or serofibrinous exudate, often existed in the absence of polymorphonuclear excess; again virus might or might not be present. Fourthly, combinations of these appearances obtained, once more with the inconstant presence of small quantities of virus; numerous polymorphonuclear leucocytes passed out into the exudate, which in the most marked instances completely filled the alveoli and small bronchi of the affected areas. The conclusion that some of the animals had, by dying, escaped pneumonia seemed unavoidable, but the exact rôle played by the virus was uncertain. More especially was this the case since in a few control animals peribronchial cuffing and even some leucocytic bronchial exudate were encountered, indicating a degree of spontaneous respiratory infection.

To summarize, in the rabbit subcutaneous, intradermal or intramuscular inoculation leads to local inflammation and necrosis followed by ascending infection of the corresponding peripheral nerve. With the onset of itching, nerve cells in the spinal ganglia and posterior horn of segments of the spinal cord corresponding to the site of inoculation undergo degeneration; acidophilic material accumulates in the nucleus to produce an inclusion of the type seen in herpetic encephalitis, cytoplasmic degeneration follows, and ultimately necrosis occurs. Similar lesions appear later in the posterior horn and spinal ganglia of the opposite side, and occasionally with long duration of symptoms in the anterior horns, but not at higher or lower levels of the nervous axis. The nerve cell degeneration is primary, and cellular reaction occurs only during the last stages of the malady. Nuclear inclusions are found also in a variety of cells in the local lesion, in sheath of

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[2] These remarks refer chiefly to experiments with the Iowa strain of virus which, in contradistinction to the Hungarian strain, does not appear in the blood in any quantity, and is inconstantly present in the lungs, spleen, etc. Even after intravenous inoculation this statement holds good.

Schwann cells of the peripheral nerve and nerve roots, in capsule cells of the spinal ganglia and in glial cells of the grey and white matter of the spinal cord.

Intracerebral inoculation is followed by the development of nuclear inclusions in mesothelial cells of the pia-arachnoid, in subpial glial cells and in superficially placed nerve cells. Though a variable degree of meningeal infiltration obtains, the nerve and glial cell lesions are clearly primary, and in places occur in the absence of any cellular reaction.

With inoculation by any route, at autopsy hemorrhages are found with some frequency in the thymus, and congestion, hemorrhage or edema, or all three changes, in the lungs. In the absence of macroscopic alterations in the lungs, the alveolar walls may exhibit marked excess of leucocytes, sometimes associated with focal proliferation of the alveolar epithelium. Virus may be present in macroscopically normal, and absent from congested and edematous lungs. Occasionally characteristic nuclear inclusions are present in proliferating alveolar epithelium.

Lesions in the Guinea Pig

Subcutaneously inoculated animals alone have been examined. Morbid changes in the nervous system are essentially the same as in rabbits, with minor differences indicative perhaps of a slightly greater degree of resistance. The visceral changes are similar to those in the rabbit.

Nuclear changes in the spinal ganglion cells developed rather more slowly. Their intensity was not wholly dependent on the duration of symptoms; in animals surviving 24 and 27 hours respectively from the onset of itching, ganglion cell destruction had progressed no further than in rabbits or other guinea pigs dying in 8 hours, and was still unilateral. A greater proportion of cells showed inclusions typical except that they were not definitely acidophilic. Capsule cells contained inclusions less frequently. Cellular reaction and neuronophagia were more pronounced; the former was, however, very variable in amount. In the most marked instance, large numbers of polymorphonuclear leucocytes, many fragmented, with some eosinophils and lymphocytes infiltrated the spinal ganglia, posterior nerve roots and posterior root entry zone; fewer were present in the tip of the posterior horn, posterior and lateral columns, and the perivascular spaces of grey and white matter. Early microglial reaction might be evident.

In the anterior horns chromatolysis and swelling of motor neurons was the rule; on occasion vacuolation also obtained. In one animal in which symptoms lasted for 56 hours, specific nuclear inclusions were present in many anterior horn nerve cells (Fig. 6), in glial cells along the line of exit of the anterior roots and in fewer numbers in the white matter generally. In cases of similarly protracted duration specific nuclear changes might exist also in the brain stem.

Lesions in the Monkey

Three monkeys (*M. rhesus*) died within 6 to 9 days of the intracerebral inoculation of Iowa virus; another was infected with the Aujeszky virus. The clinical and experimental data will be considered in a future paper. A fifth monkey died of pulmonary tuberculosis towards the end of the incubation period (4th day). The histological picture differed in important particulars from that in rodents.

The lesion in the monkey consisted in widespread, primary degeneration of nerve cells, which showed various changes accompanied by increase of intranuclear acidophilic material, or appeared as shrunken, eosinophilic, necrotic structures with or without pyknotic or fragmented nuclear remains (Fig. 7). Similar changes accompanied by clasmatodendrosis occurred in fewer neuroglial cells, while others showed some enlargement of the cell body. In many regions no other changes were present. Where from greater severity, or, as experience with the 4 day animal suggested, from longer duration of the infective process, a larger proportion of nerve cells was involved, early diffuse microglial reaction and occasional small focal collections of these cells were observed. With still more intense lesions a few polymorphonuclear leucocytes, often fragmented, appeared in the nervous tissue. In such areas a proportion of the neurons exhibited degenerative changes (swelling, solution of the Nissl substance, vacuolation, shrinkage and basophilic impregnation, impregnation of the pericellular Golgi net, etc.) without specific nuclear changes. It could not be decided whether all the necrotic elements had passed through an inclusion-bearing stage or not. Evidence of neuronophagia was occasionally present.

Stained with phloxin-methylene blue the nerve cell inclusions appeared as (*a*) aggregates of comparatively few, coarse, pale pink granules or of many finer granules, (*b*) irregular, deeper pink masses like those described in the rabbit, (*c*) multiple deep pink spherules recalling the bodies in experimental poliomyelitis (Covell, 1930, Hurst, 1931) and, to a less extent, those in Borna disease. Combinations of these were encountered. Their formation was accompanied by early fragmentation of the nucleolus and margination of the basophil chromatin. Glial cell inclusions were less common and mainly of the first variety; they were present chiefly in the grey matter, and only rarely in neuroglia or oligodendroglia of the white matter or in ependymal cells. Inclusions were never, as in the rabbit, present in mesodermal elements of the vessels or meninges.

Meningeal infiltration with polymorphonuclear leucocytes and lymphocytes was intense only over the site of inoculation and more marked over the remainder of the inoculated hemisphere than on the opposite side, where it might be wholly wanting. It was much less pronounced in passage animals than in the monkeys infected with rabbit brain. Only once did the infiltration extend to the brain stem and upper part of the spinal cord. Where the process was less marked, the cells collected chiefly in the walls of the meningeal veins and in the depths of the Sylvian fissure, superior temporal sulcus and cingular sulcus.

Save for slight polymorphonuclear and lymphocytic cuffing in severely affected cortical areas, perivascular cuffing was marked in only two situations, in the tissues around the third ventricle along the vessels entering the anterior and posterior perforated spaces, and in the grey matter surrounding the ventricular system of the brain stem. Occasionally a few infiltrated vessels occurred in the white matter of the hemispheres. In one animal perivascular infiltration was wholly wanting.

Distribution of Lesions.—At the site of inoculation, edema, limited necrosis and hemorrhage were accompanied by reparative changes without specific inclusions in the newly formed tissue. Lesions in the cortex were not determined primarily by adjacence to the inoculated area, and varying degrees of change obtained in gyri equidistant from this.

No major subdivision of the cerebral cortex appeared immune from attack, and there was no tendency to selective involvement of any cortical layer. On the whole, changes were much less marked at the frontal and occipital poles, and at the vertex than at the base: once, however, the vertical cortex was most severely damaged. At the vertex, lesions were usually most intense in the vicinity of the cingular sulcus, on the lateral surface in the island of Reil and neighbouring cortex. Here the proportion of cells affected commonly reached 20–50 per cent of the total. In all but one animal the basal surface of the frontal lobes and the anterior temporal cortex (superior and middle temporal gyri) were most severely damaged; just posterior to the temporal pole from 80–100 per cent of the nerve cells had perished or were severely injured. In two animals extensive destruction continued into the pyriform area, cornu Ammonis and dentate gyrus, with almost complete disorganization of structure. The findings in the 4 day animal suggested an earlier affection of the lower frontal and temporal than of the remaining cortex. Lesions were more intense on the inoculated side.

Compared with the cerebral cortex, the optic thalamus and globus pallidus were lightly affected, while the caudate nucleus and putamen escaped damage or exhibited only mild changes. The hypothalamic region and tissues around the third ventricle were affected with moderate severity; here perivascular infiltration and focal microglial proliferation, relative to the amount of nerve cell involvement, were more marked than elsewhere.

In the midbrain and pons perivascular infiltration was marked in the grey matter around the aqueduct of Sylvius and in the floor of the fourth ventricle. Isolated nerve cells, or occasionally the majority of cells in a particular nucleus, showed specific changes with focal microglial reaction in the surrounding tissue. Nerve cells not showing nuclear inclusions were always perfectly preserved. Changes were distinctly more frequent in the dorsal than in the ventral regions. Similar lesions occurred in diminishing intensity through the medulla to the upper cervical segments of the cord. The cerebellum, lower cord, Gasserian and spinal ganglia and sciatic nerves were intact.

The other viscera showed no definite abnormality, except the salivary glands in which a certain number of acini manifested the results of marked secretory overactivity.

In the monkey, therefore, the essential lesion following intracerebral inoculation is widespread, primary degeneration of nerve and glial cells, chiefly in the cerebral cortex (of both sides), and attaining its maximum in the first and second temporal gyri, pyriform area, cornu Ammonis and island of Reil. The absence of nuclear inclusions from mesodermal elements in the meninges and elsewhere suggests that in this animal the attack of the virus is directed solely against ectodermal structures, which is far from being the case in the rabbit. Cellular reaction in the cerebral cortex is clearly secondary to the nerve cell necrosis. Perivascular infiltration is most marked in the grey matter around the third and fourth ventricles, where nerve cell damage is relatively slight. Meningeal infiltration may be wholly absent from the uninoculated hemisphere, and is much less pronounced in passage animals than in those infected with foreign (rabbit) nervous tissue; evidently in the latter non-specific factors play a part in its production. No significant lesions develop in other viscera.

Lesions in the Cow

From the scanty material available it appears that lesions in the cow approximate more closely to those in the monkey than to those in the rabbit.

Through the courtesy of Dr. Shope, a few pieces of brain from the cow furnishing the original strain of mad itch virus were available for examination.

At all levels of the cord, brain stem and basal ganglia, and in one part of the cerebral cortex, pathological changes were present. Perivascular infiltration, of moderate degree, with large and small lymphocytes and occasional large mononuclears was associated with areas of semidiffuse microglial proliferation in the grey matter; isolated polymorphonuclear leucocytes and lymphocytes were present in the nervous substance. In the white matter smaller compact microglial foci were of rare occurrence. The majority of nerve cells were normal or in a condition of mild chromatolysis; a few were acutely necrotic or, very rarely, undergoing neuronophagia. In a minority nuclear inclusions were visible; the condition of the material was not ideal for fine cytological study. At one level of the medulla scanty meningeal infiltration with mononuclear elements was noted.

Lesions in the Pig

Intracerebral Inoculation.—In three pigs dying 3 1/2, 4 and 5 days after intracerebral inoculation the histological appearances differed considerably from those hitherto described. Vascular and interstitial changes were most conspicuous, and damage to nerve cells was relatively slight.

Two animals received rabbit brain as inoculum; in the brain of one of these meningitis was everywhere intense. In the second, and in that of an animal infected with pig brain, meningitis though fairly generalized was definitely less marked; it was severe only over the base and upper medial surfaces of the hemispheres and over the cerebellum. In all, the infiltrating cells were largely lymphocytes with a notable number of eosinophils, a few large mononuclears and rare polymorphonuclear leucocytes and plasma cells.

In the nervous tissue, the congested vessels of both grey and white matter frequently showed cuffing with a single layer of similar cells. In some parts of the cortex, particularly in the areas of most intense meningitis, perivascular infiltration was more marked and often associated with diffuse proliferation of microglia in the surrounding tissues (Fig. 8); microglial proliferation and cellular infiltration also occurred in the superficial cortical zone immediately beneath the pia-arachnoid. Some large foci of unusually densely packed microglial cells, with lymphocytes and eosinophils, occurred independently of the vessels; many of these cells were karyorrhectic. In stained sections such foci were readily visible to the naked eye. Smaller foci, more comparable with those commonly seen in virus infections, occurred occasionally in the white matter. Identical vascular and interstitial changes obtained around the whole ventricular system and in the choroid plexuses.

By comparison with the foregoing the nerve cell changes were slight, and relative to those in the monkey, insignificant. Only in the densest tissue foci did some neurons manifest severe degenerative phenomena culminating in death and neuronophagia. Elsewhere they exhibited mild swelling and chromatolysis of such general distribution as to suggest the uniform action of a toxin rather than that of a virus. Neuroglial nuclei were often swollen and hydropic.

The surprising feature in the pig was, however, the complete absence of typical nuclear inclusions such as were found in all other animals. In some swollen glial nuclei scanty oxyphilic material was sometimes present. It was not possible to deny that this might have been the homologue of the typical inclusions present in other species, but it could hardly be demonstrated as a convincing example of this type of nuclear degeneration.

Distribution of Lesions.—Edema was pronounced in a wide zone around the site of inoculation. The relatively slight cortical changes were as marked in the occipital region as elsewhere. In the frontal region the olfactory cortex was most heavily involved. The caudate nucleus, putamen and optic thalamus suffered less severely than the globus pallidus. The hypothalamic region and tissues around the third ventricle were markedly affected.

In the brain stem the dorsal region suffered more than the ventral; in both, extensive areas of diffuse microgliosis or more circumscribed foci might be observed. In the cerebellum similar foci involved all layers of the cortex. In the spinal cord foci occurred in both grey and white matter. Finally, in one case, mononuclear and eosinophilic infiltration extended into the optic nerve, and in two cases, along the fifth nerve as far as the Gasserian ganglion.

Subcutaneous Inoculation.—The pig differs from other animals in that, after subcutaneous inoculation, it passes through a mild febrile illness unaccompanied by itching, and only rarely develops nervous symptoms (Shope, 1931, 1932). Yet the local lesion is almost as pronounced as in other animals, and definite changes are detectable in the nervous system.

On the 6th day after inoculation in a pig recovering without having shown nervous symptoms, foci of necrosis in the corium were associated with extensive edema, fibrinous exudate and polymorphonuclear infiltration; around this area was a wide zone in which mononuclears gradually replaced polymorphonuclear leucocytes as the predominant cell type. The vessels were surrounded by many layers of similar cells; the large arteries and veins exhibited pronounced inflammation with cellular infiltration of all their coats and considerable proliferation of their endothelial linings (Fig. 9). The nerve bundles were often buried in cellular exudate and inflammatory cells lay between the individual fibres. Again no definite nuclear inclusions were demonstrated.

The corresponding spinal ganglia showed heavy polymorphonuclear and mononuclear infiltration, together with ganglion cell degeneration sometimes culminating in acute necrosis with neuronophagia by capsule cells or lysis by polymorphonuclear leucocytes. Infiltration continued along the nerve root into the cord, where microglial foci in grey and white matter and perivascular infiltration obtained together with mild chromatolysis of the nerve cells. At other levels of the cord, in the brain stem and in the cerebral hemispheres many vessels were cuffed with a single layer of lymphocytes, and occasionally foci of inflammatory cells occurred in the meninges. Meningeal infiltration was rather more marked over the cerebellum, with some microglial reaction in the nervous tissue immediately subjacent. Everywhere in the brain the nerve cells were well preserved.

Lesions in Other Organs.—In one of the two intracerebrally inoculated pigs in which the general viscera were examined, the cervical lymph glands were enlarged and showed macroscopic hemorrhages at the periphery. Microscopically, many eosinophils infiltrated the lymphoid nodules, particularly at the periphery of the gland, and numerous extravasations of blood were present in the relatively acellular zone between the lymphoid tissue and the capsule; the capillary endothelium was obviously swollen. Many eosinophils were present in the spleen, again mainly in the lymphoid nodules. In the second pig, numerous polymorphonuclear leucocytes and eosinophils occupied the acellular zone; no hemorrhage or endothelial swelling was noticed.

In the subcutaneously injected animal, frank necrosis with considerable focal infiltration of polymorphonuclear leucocytes occurred in the same situation and in the processes of poorly cellular tissue penetrating towards the centre of the gland. In this animal foci of necrosis obtained also in the lymphoid nodules of the spleen. The heart showed acute myocarditis; the muscle bundles were separated by edema and numerous polymorphonuclear leucocytes were present in the tissue.

In the pig after intracerebral inoculation, meningeal, perivascular and tissue infiltration is far more pronounced than in the rabbit, cow or monkey. On the other hand, nerve cell damage is comparatively slight. Surprisingly enough no definite intranuclear inclusions are demonstrable.

After subcutaneous injection local inflammation and necrosis occur. Heavy cellular infiltration in the corresponding spinal ganglia is accompanied by nerve cell degeneration and occasionally necrosis. No inclusions are seen in either situation. In the spinal cord lesions are less severe. In the brain mild perivascular infiltration is evident.

The involvement of the lymphatic system constitutes a further difference in reaction in the pig.

Symptomatology of the Disease in the Rabbit Considered in the Light of Histological and Experimental Findings

Cause of Itching.—As already mentioned, 24 hours after subcutaneous or intramuscular injection, inflammation is already well advanced locally, and inclusions are present in connective tissue and other cells. Within 40 hours necrosis is evident, and lesions are extending proximally along the peripheral nerve leaving the damaged area. Itching does not begin for 50 hours or more (with the Iowa virus). During the period of irritation a saline extract of the local lesion inoculated into a fresh animal evokes no immediate symptoms. If inoculation is practiced in the calf muscles, provided that care be taken to prevent leakage into the overlying skin, itching is not local but is referred to the terminal distribution of the sciatic nerve, and perhaps to the posterior part of the flank of the affected side. These facts suggest that the symptom is not due to irritation of nerve endings at the site of inoculation.

On the other hand, after subcutaneous or intramuscular injection, itching commences about the time when lesions can first be demonstrated in the corresponding spinal ganglia and segments of the spinal cord, and about 2–3 hours after virus can first be detected there. Although virus can later be detected in smaller amount at higher levels of the cord, no lesions are ordinarily present at these levels, and no itching occurs in the corresponding peripheral area. In about half the cases, intravenously inoculated animals itch at some point (unrelated to the site of venepuncture), and lesions are then present in the corresponding spinal ganglia. After intracerebral injection early lesions are occasionally present in the Gasserian ganglion; once an animal so inoculated scratched the face violently, but unfortunately the ganglion was not examined. In short, very early lesions, or scanty lesions affecting only occasional cells, may appear in spinal ganglia or cord without signs of peripheral irritation; otherwise there seems to be perfect correlation between the two phenomena.

Cause of Respiratory Symptoms and of Death.—Towards the end of the illness following subcutaneous or intramuscular injection, breathing is usually rapid and shallow, and death takes place from respiratory failure. After intravenous inoculation about half the animals do not itch; these may manifest unrest, convulsions, general subsultus, and rapid or laboured breathing, or may die suddenly without symptoms having been observed. Shope records similar lack of symptoms with nasal inoculation. Visible pathological lesions in the lungs are by no means always present when definite respiratory distress has been noted.

Now virus is invariably present in the medulla at the time of death, though usually in low concentration, giving a long incubation period in the passage animal. It has previously been remarked that no lesions are demonstrable here. In those intravenously inoculated animals which do not itch, no lesions are found in the central nervous system. These facts suggest that the virus may have reached the medulla only shortly before death, a suggestion supported by the data given in Table I. In the guinea pig symptoms may exceptionally endure for as long as 30–50 hours; then early changes are present in the medulla. We may probably infer that the medullary centres of the rabbit are peculiarly susceptible to the virus, and that only a minimal amount acting for a short time is necessary to arrest their function. Under suitable conditions, therefore, *e.g.* after intravenous inoculation, death may immediately follow infection of the medulla before specific lesions have time to develop. On these grounds it seems that the name "infectious bulbar paralysis" introduced by Marek (1904) ideally designates the malady in rabbits.

TABLE I

Time of Appearance of Virus in Medulla of the Rabbit Following Inoculation into the Leg

No.	Duration of symptoms	Killed or died	Presence of virus in medulla
	hrs.		
53	0	K	0
54	1	K	0
55	3	K	0
56	6	K	0
57	8	K	0
58	8	D	+82
59	10	K	0
63*	10	D	+78
47*	10	D	+105
60	11	K	0
61	11	K	+126
62	14	D	+110
64	18	D	+105

+ = development of pseudorabies in passage animal with incubation period in hours.

0 = no take.

* Inoculation into flank.

DISCUSSION

The virus of pseudorabies is capable of exciting lesions in a wide range of animal hosts; in all it exhibits definite predilection for the nervous system. Yet while it may be grouped with the neurotropic viruses, it does not behave as a strict neurotrope since in some animals at least it produces specific changes in non-nervous tissues. Thus, in the rabbit, inclusions may be found in a great variety of cells, a probable indication of ability on the part of the virus to parasitize cells derived from any embryonic layer. In these respects the organism bears considerable resemblance to the herpes virus.

Comparative study of the lesions evoked in different animal species affords a striking example of differences in host reaction to a single infective agent. In the rodent, the monkey and the cow, nerve cell degeneration is clearly primary and independent of vascular and interstitial inflammation, though in all reactive changes occur sooner or later. In the cow and the monkey nuclear inclusions do not occur, as in the rabbit, in other cells besides nerve and glial cells. In the pig the meningeal and vascular reaction is dominant. In this animal, in the absence of clear cytological evidence of direct virus attack on the comparatively intact nerve cells of the brain, it is difficult to assess the relative parts played by the virus and by impaired nutrition consequent upon these other factors. The amount of nerve cell degeneration in the spinal ganglia of the subcutaneously inoculated pig was undoubtedly too great to be attributed solely to vascular and interstitial lesions; again, however, no nuclear inclusions existed as a mark of the activity of the virus. (Whether the porcine cell is capable of reacting in the particular manner necessary to develop intranuclear inclusions is not known. There is no *a priori* reason to suppose that it is not, but such bodies have never been convincingly demonstrated in the pig.) In the pig, too, the obvious involvement of the lymphatic system constitutes a further difference in reaction to the infection. It would appear that, from clinical and pathological viewpoints alike, pseudorabies in the pig is very different from the disease in most animal species.

During the incubation period of the disease following inoculation into the leg of the rabbit, the extending chain of nuclear inclusions along the branches of the sciatic nerve indicates a route followed by the virus.[1] Moreover, from the presence of these inclusions in the cells of the sheath of Schwann, and the variable degree of inflammatory

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[1] In a future publication evidence will be presented that the virus can spread also by other channels; the distribution of lesions described above leaves no doubt, however, that the nervous route is the most favourable.

reaction in the neural connective tissue sheath, it appears likely that the virus exists interstitially in the nerve and is not only passing along the axis-cylinders. On the other hand, at a time when early lesions appear in the corresponding spinal ganglia, and slightly later when inclusions are becoming relatively numerous in the posterior nerve root between the ganglion and cord, inclusions in the upper part of the sciatic nerve are still very few in number; a possible explanation, for which there is at present no supporting evidence, is that virus also travels, and travels more quickly, by the axis-cylinders and becomes liberated interstitially at the level of the ganglion. Although in cases of protracted duration, and therefore more especially in the guinea pig, nuclear inclusions may be found in the anterior horn nerve and glial cells, their appearance here follows by a definite interval their development in the spinal ganglia, posterior roots and posterior horn. This may indicate a greater susceptibility of the sensory neurons or, if the virus can traverse the axis-cylinders, that the direction of the nervous impulses may affect its speed of progression. After intracerebral inoculation in the rabbit, the distribution of inclusions in nerve and glial cells is largely explicable by assuming a meningeal spread of the infection. In the monkey too, the relative severity of the lesions closely parallels the quantitative distribution of dyestuffs injected into the cisterna magna (Hurst, 1932); if, however, in this animal the virus spreads by the meninges, it does not necessarily leave any trace, since in the uninoculated hemisphere severe nerve cell destruction may occur in the temporal lobe, etc., in the complete absence of meningeal infiltration. Reference has already been made to the prominence of meningitis in the pig.

Intracerebral lesions in the rabbit differ sufficiently from those of herpes to permit of their differentiation. In herpes, meningitis may be pronounced, but the nuclear inclusions in mesodermal elements are much less numerous, and the subpial glial cells are not affected with the same frequency or over as wide an area as in pseudorabies. Eosinophils and lymphocytes play (at the time of death) a more, and polymorphonuclear leucocytes a less important part in the infiltration. Involvement of the nervous tissues is more marked, with abundant necrosis of nerve cells and much more cellular infiltration around the vessels and in the tissues. Nuclear inclusions, while of the same general type as in pseudorabies, tend to be more coarsely granular and more often fill and distend the nucleus.

SUMMARY

The histology of pseudorabies differs materially in various animal species. In the rabbit, subcutaneous, intradermal or intramuscular inoculation leads to local inflammation and necrosis. The infection ascends the peripheral nerve (possibly both interstitially and by the axis-cylinders) to the corresponding spinal ganglia and segments of the spinal cord, where primary degeneration of nerve and glial cells takes place. The nerve cell changes are probably responsible for the cardinal symptom of the disease, itching. Death ensues soon after virus reaches the medulla, before visible changes have been produced here. Intracerebral inoculation is followed by characteristic lesions in the meninges, in subpial glial cells and in superficially placed nerve cells. Morbid changes in the lungs are not necessarily related to the presence of virus, but specific lesions may be present. Intranuclear inclusions bearing some resemblance to those in herpetic encephalitis, yellow fever, etc., occur in cells derived from all embryonic layers.

The disease in the guinea pig resembles closely that in the rabbit and is modified only by the slightly greater resistance of the animal.

In the monkey after intracerebral inoculation, widespread degeneration and necrosis of cortical nerve cells are accompanied by the appearance of specific nuclear alterations in nerve and glial cells, but not in cells of mesodermal origin. No lesions are found in other viscera.

In the spontaneous disease in the cow lesions approximate more closely to those in the monkey than to those in the rabbit.

In the pig vascular and interstitial lesions predominate, nerve cell degeneration is relatively slight and typical inclusions are not observed. These differences probably explain the benign course of the malady following subcutaneous inoculation in this animal. The lymphatic system, too, participates in the reaction to the virus.

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EXPLANATION OF PLATES

PLATE 20

FIG. 1. *A*. Normal spinal ganglion cell of rabbit. The nucleus contains a prominent nucleolus and a variable amount of granular, weakly acidophilic material enclosing one or more rather definite, more strongly acidophilic masses ("nucleonephelium" of Saguchi). The peripheral cytoplasmic zone free from Nissl bodies is a normal feature.

B–G. Successive stages of degeneration of spinal ganglion cells following subcutaneous inoculation of pseudorabies virus; acidophilic nuclear degeneration leads to formation of intranuclear inclusions of the type seen in herpetic encephalitis.

B. Definite increase of acidophilic intranuclear material grouped chiefly around the nucleolus leaving most of the nucleus empty. The Nissl substance is perfectly preserved.

C. More pronounced increase of acidophilic material. The nucleolus has disappeared. The Nissl bodies are still normal.

D. The nucleus is filled with finely granular, feebly acidophilic material enclosing more strongly acidophilic aggregates. The nucleolus has disappeared and the Nissl bodies are disappearing.

E. The nucleus is completely filled with acidophilic material; fragments of the nucleolus are present. The Nissl substance has entirely disappeared. Inclusions are seen in the nuclei of some capsule cells.

F. Acidophilia of the cytoplasm and a shrunken nucleus indicate death of the nerve cell. Inclusions are present in some of the capsule cells.

G. Neuronophagia by proliferated capsule cells, some bearing nuclear inclusions, an unusual picture in the rabbit. A few karyorrhectic polymorphonuclear leucocytes are present.

H. Nuclear inclusions in mesothelial cells of the pia-arachnoid and subpial glial cells following intracerebral inoculation in the rabbit. Pyknotic remains of infiltrating leucocytes are also seen.

Drawn from preparations stained with phloxin-methylene blue after sublimate-formol or Zenker-formol fixation.

PLATE 21

FIG. 2. Spinal ganglion of rabbit showing great majority of nerve cells in various stages of the degeneration described in the text. The darker cells are necrotic. Sublimate-formol: phloxin-methylene blue. × 172.

FIG. 3. Early changes in one spinal ganglion cell of rabbit (above): later stage (below) with intranuclear inclusion in a capsule cell. Sublimate-formol: phloxin-methylene blue. × 1000.

FIG. 4. Inclusions in mesothelial cells of pia-arachnoid in rabbit: intracerebral inoculation. Sublimate-formol: phloxin-methylene blue. × 445.

FIG. 5. Inclusions in Purkinje cell of cerebellum and adjacent glial cells. Intracerebral inoculation in rabbit. Sublimate-formol: phloxin-methylene blue. × 1165.

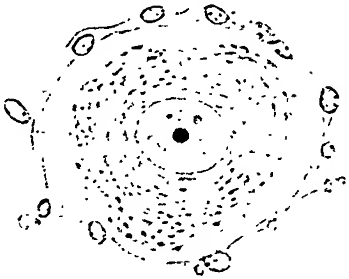
PLATE 22

FIG. 6. Nuclear inclusion in anterior horn cells of guinea pig (above and to left). Zenker-formol: iron alum hematoxylin and eosin. × 455.

FIG. 7. Reactionless acute necrosis in cornu Ammonis of monkey: intracerebral inoculation. Sublimate-formol: iron alum hematoxylin and eosin. × 325.

FIG. 8. Perivascular and tissue infiltration in cerebral cortex of pig: intracerebral inoculation. Sublimate-formol: iron alum hematoxylin and eosin. × 245.

FIG. 9. Pronounced arteritis with endothelial proliferation in skin of pig following subcutaneous inoculation. Bouin: iron alum hematoxylin and eosin. × 160.



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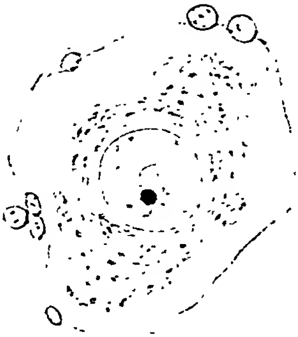

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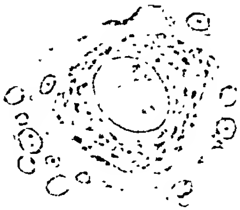

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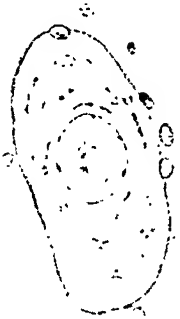

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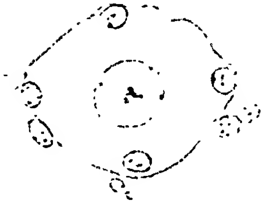

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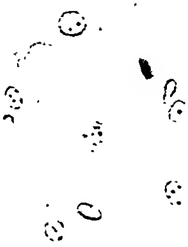

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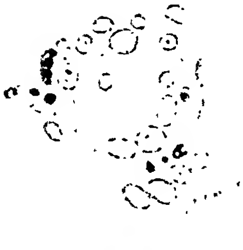
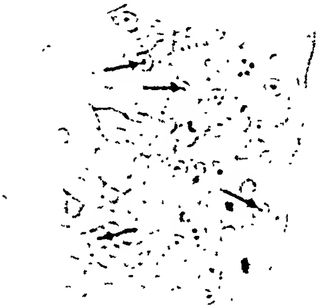
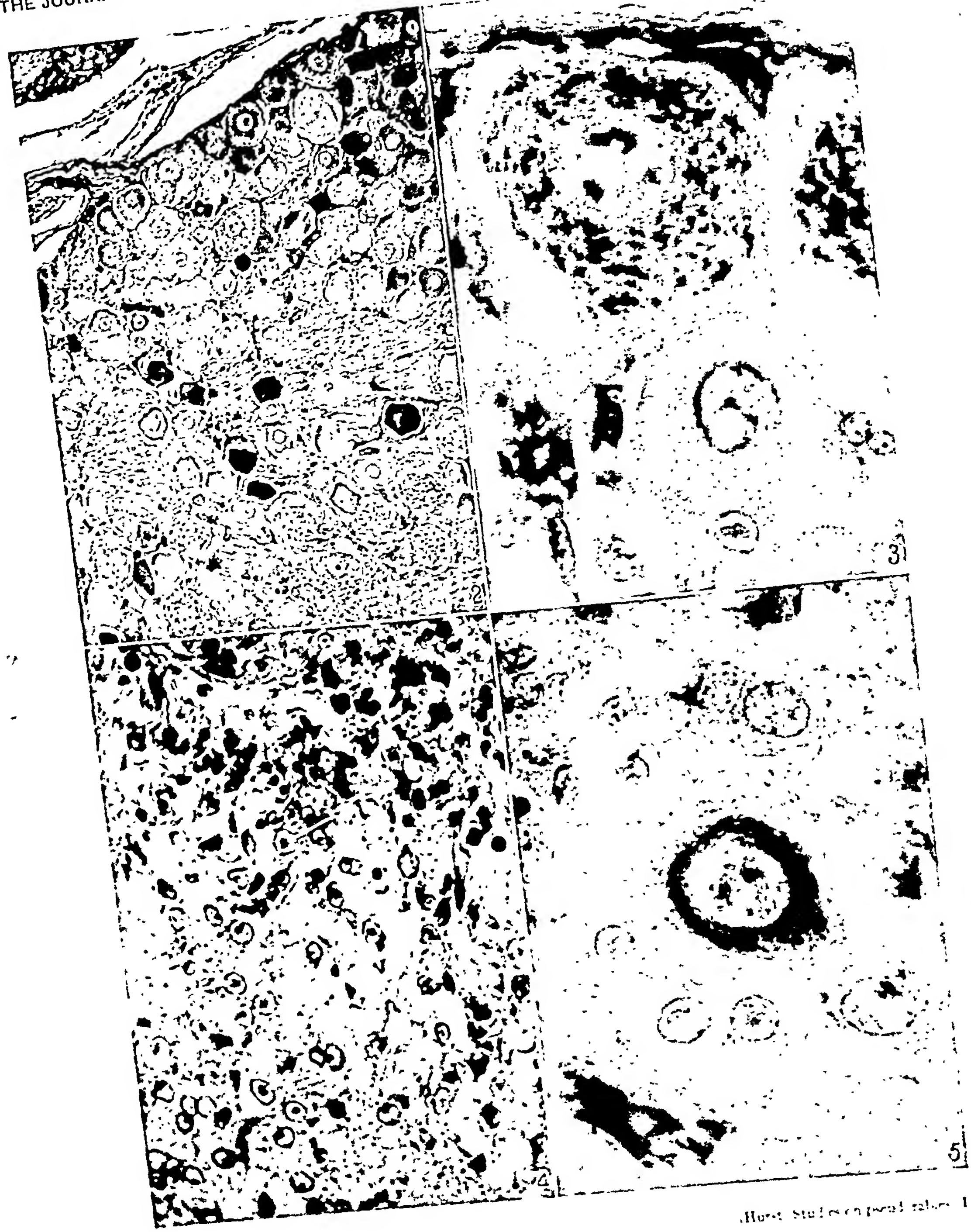
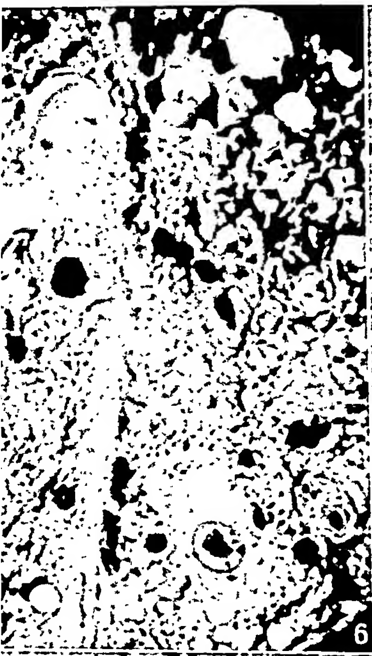






(Hurst: Studies on pseudorabies. I)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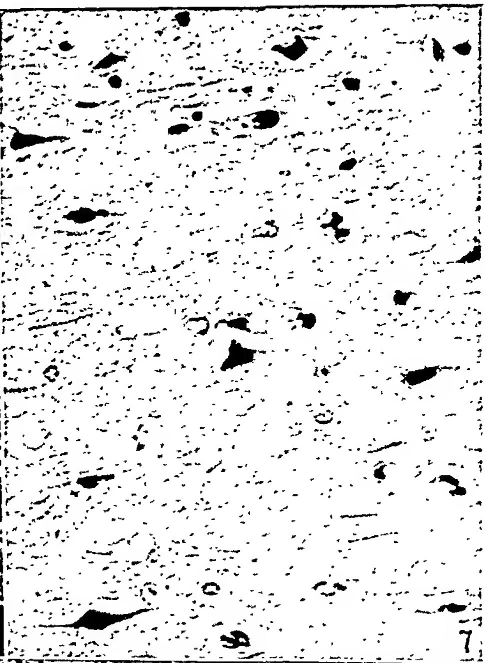
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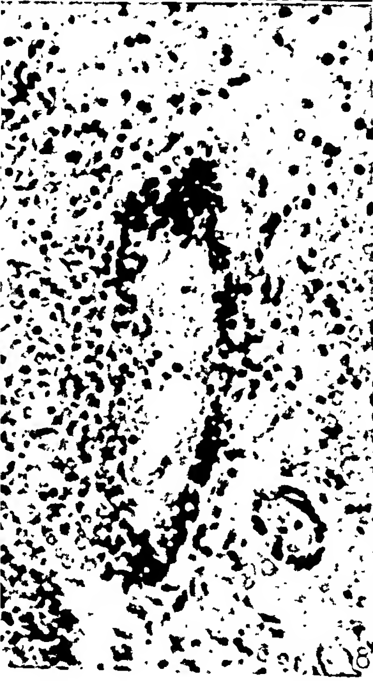
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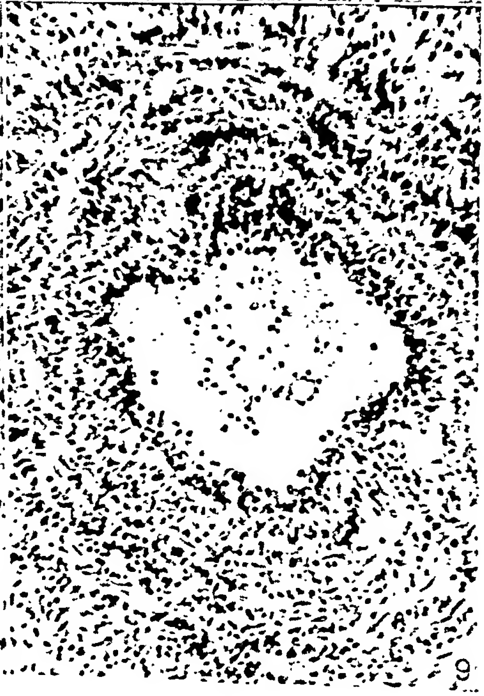
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(Hurst: Studies on pseudorabies. I)

SOME OBSERVATIONS ON THE SPECIFICITY OF BACTERIAL ALLERGY TO CERTAIN OF THE NEISSERIAE

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Of the phenomena manifesting hypersensitiveness to bacteria or their products, the cutaneous reaction has received the greatest attention of investigators. The first example to be carefully studied was the tuberculin reaction, which was readily applied as a practical diagnostic procedure by the clinician and the veterinarian. The analogs of the tuberculin test: the typhoidin, mallein, luetin, abortin, gonococcin reactions, are similar examples of exaggerated inflammatory response in the skin of a host to the introduction of organisms with which it (the host) is infected.

Reports of the clinical application of this technique do not here concern us, and of the literature on the experimental work on the problem only a few studies need be cited; *viz*, those which have inquired into the underlying mechanism of the reaction and those bearing on its specificity. Zinsser and his coworkers have published several papers on the relation of bacterial allergy, as demonstrable by the cutaneous reaction, to anaphylaxis. A summary of his views is contained in a lecture delivered Jan. 5, 1928, before the New York Academy of Medicine (1). Using the method of Zinsser and Raymond (2) for producing chronic pyogenic foci in guinea pigs, Zinsser and Parker (3) studied the allergic cutaneous reaction to staphylococci. They also sensitized guinea pigs to typhoid bacilli by repeated intracutaneous inoculation. The cross-reactions which they encountered will be discussed below.

MacKenzie and Woo (4) found that guinea pigs repeatedly injected intracutaneously with pneumococcus protein developed during the 3rd week a hypersensitiveness which continued for about a week and then disappeared. The animals, however, showed no alteration in their susceptibility to intraperitoneal inoculation with pneumococci.

Julianelle and Avery (5) found that rabbits repeatedly injected intracutaneously with heat-killed pneumococci developed typical allergic reactions to pneumococcus nucleoprotein, but not to the soluble specific substance—the type-specific carbohydrate of Avery and Heidelberger. Intravenously vaccinated animals failed to become allergic.

Zinsser and Grinnell (6) reported the production of allergy to hemolytic streptococci by means of agar foci infected with living organisms. The most extensive study of streptococcal allergy, however, has been made by Swift and his coworkers. Their interest in this phenomenon grew out of the observation of Andrewes, Derick, and Swift (7) that about half of their rabbits which had been inoculated intracutaneously with non-hemolytic streptococci suffered a recrudescence of the local inflammatory process 8 or 9 days after the inoculation. This was found (8) to be due to the development of a state of hypersensitiveness (analogous to that which evokes the tuberculin reaction) and has been studied in great detail by Derick and Swift (9). Intravenously immunized animals responded to intracutaneous injection by the formation of small hard nodules, quite different from the large, acutely inflamed lesions which appeared in the sensitized animals (10). Sensitization was effected by a variety of procedures (11), so long as small numbers of organisms were introduced at a time or a very low grade, chronic infection maintained.

In the literature on this subject of cutaneous hypersensitiveness to bacteria the specificity of the reaction seems to have been rather assumed than demonstrated. For with few exceptions mention is not made of reactions to organisms other than the ones with which the animals had been sensitized. In their recent study of the bacterial endotoxin of *Salmonella pullorum* Hanks and Rettger (12) mention the non-specificity of cutaneous reactions to *Salmonella pullorum*, *Proteus vulgaris*, and *Serratia prodigiosus* in rabbits rendered hypersensitive to any of these organisms.

In a study of the abortin reaction Stroem (13) states that he was unable to sensitize guinea pigs to heat-killed cultures of *B. abortus*, even when they were mixed with kieselguhr to stimulate local tissue reaction. He did observe cutaneous hypersensitivity in animals infected with *B. abortus* in which gross anatomical lesions were entirely absent. Tuberculous animals were found to give slight reactions to abortin, and he attributed this to heightened, non-specific reactivity.

Hanger (14) found that rabbits (presumably the same ones which he mentions as carriers of *Bact. lepisepticum* in their upper respiratory passages) reacted to intradermal inoculation with filtrates of *B. influenzae*, *B. coli*, and meningococcus as well as of *Bact. lepisepticum*. He also states that human beings who react to filtrates of the aforementioned organisms also react to those of *Bact. lepisepticum* and he concludes, therefore, that "there is apparently considerable antigenic relationship between many Gram-negatives of different biological groups."

The study here reported took its origin from an interest in the interrelation of certain of the Gram-negative diplococci (15) but gradually grew in scope by the inclusion of unrelated organisms, largely for purposes of control. Since we had at our disposal a stock of snuffle-free rabbits[1] we were able to repeat Hanger's observations without the complicating factor which he mentions, the presence of *Bacterium lepisepticum* in the upper respiratory passages of the rabbits.

Methods and Materials

The observations reported below were all made on young adult rabbits from a snuffle-free stock protected at all times from possible contact with carriers of this disease.

Cultures.—The Neisseriae employed were gonococcus (6 strains), meningococcus (2 strains) and *Micrococcus catarrhalis* (1 strain), as well as *Bact. lepisepticum*,[2] and R pneumococcus originally derived from a Type I organism, and in a few instances *Staphylococcus aureus*, and scarlatina streptococcus. Except as noted to the contrary, the organisms were grown for 18 hours on an egg white digest agar similar to that described as control medium in a preceding publication (16). The liquid medium had the same composition as the solid less the agar.

Agar Foci.—Large tubes of 2 per cent agar were melted and cooled to about 45°C. The appropriate inoculum of organisms was then mixed with 20 cc. of melted agar and at once injected under the skin.[3] The regions usually selected were the outer surface of the upper leg or the flank near the rib margin. A long needle was used so that the agar was placed at some distance from the puncture wound. Pieces of ice were held about the margin of the injected mass to cool it quickly and prevent its spreading out into a thin layer and also to prevent its leaking back through the tunnel made by the needle.

Most of the rabbits tolerated their foci with little apparent difficulty. Some of them, particularly those bearing foci heavily infected, became emaciated and had to be sacrificed before they could be tested by intracutaneous inoculation.

The skin overlying the focus partook of the inflammatory reaction which

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[1] This supply of snuffle-free rabbits was made available by the generosity of Harold H. Swift, Esq., who raised them under unusually favorable conditions at his farm near Lakeside, Mich. The original stock was obtained through the courtesy of Dr. Leslie T. Webster of The Rockefeller Institute for Medical Research and the late Professor Carroll G. Bull of the School of Hygiene and Public Health of the Johns Hopkins University.

[2] The strain of *Bact. lepisepticum* was very kindly sent to us by Dr. Leslie T. Webster of The Rockefeller Institute for Medical Research.

[3] This method seems to have been introduced by Dochez, who employed it for the immunization of horses to the toxins of scarlatina streptococci. It was adapted to the use which here concerns us by Swift in his experiments (referred to above) on the sensitization of rabbits to non-hemolytic streptococci. The procedure of Zinsser and Raymond was to fill a celloidal sphere with melted agar containing live organisms and place it in the peritoneal cavity of an animal.

developed about it, and, when the focus had been too superficially placed, underwent necrosis with resulting extrusion of the remnant of the agar mass. This occurrence, which was infrequent, did not seem to impair detectably the rabbit's development of hypersensitiveness, possibly because it did not take place until several days after the implantation.

The inflammatory reaction was appreciably more marked about foci containing gonococci and meningococci than around those containing *M. catarrhalis.*

Microscopic and bacteriological study was made only of foci containing gonococci. In these the sequence of events may be briefly described as follows: Within an hour or two after implantation, invasion by polymorphonuclear leucocytes begins. These cells ingest the bacteria which they encounter and in doing so are damaged or killed. But enough of them enter to constitute a steadily—and quite rapidly—advancing border which is followed by a second containing other phagocytic cells and a third consisting of capillary loops, which make their appearance on the 2nd or 3rd day. Section of a focus at this time or later reveals concentric rings of these elements. Gonococci can be cultivated from the center of a focus only so long as it is beyond the reach of the polynuclear leucocytes.

Eventually the periphery becomes encapsulated, the interior more or less liquified for a time, and as organization proceeds, the whole mass gradually contracts to a firm cyst-like structure with a caseous center.

Preparation of the Rabbits' Skins.—Epilation was accomplished by the use of impure barium sulfide[4] as recommended by Derick and Swift (9).[5] The ventral surface of the abdomen was alternately washed with soap and water and rinsed in warm running tap water several times to cleanse the fur of oil so that the barium sulfide could quickly penetrate to the skin. 10 to 15 gm. of the powdered barium sulfide were then dusted on to the fur and gently patted with a large pledget of cotton soaked with water. After a few seconds the rabbit was held under the tap and very throughly washed to remove all of the barium sulfide. The hair was removed by the stream of water. The animal was then dried by patting the epilated skin gently with a towel and rubbing the rest of the fur. The skin was then greased with vaseline. Epilation was usually performed 1 day before the intracutaneous tests were made so that areas which had been irritated by the process might be avoided in placing the inoculations. After a little practice, however, it is possible by this method to remove the hair from rabbits without doing any noticeable damage to the skin.

Intracutaneous Inoculations.—Injections of 0.1 cc. were made into the substance of the skin itself by means of a very fine hypodermic needle and a tuberculin syringe. The suspensions of organisms were standardized by Gates' method (17), the density being such that the ring just disappeared at a depth of 1 inch

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[4] We use Merck's preparation labeled "barium sulphide, gray powder, about 80 per cent."

[5] Derick and Swift (9), page 619.

(when checked by actual counts found to contain about 6.5×10^5 organisms per cc.). This simple method of standardization sufficed in these experiments because control animals were always injected with identical inocula and the relative, not the absolute, sizes of the resulting lesions were taken into consideration in judging the results of any experiment.

In the case of the Gram-negative diplococci, 18 hour cultures freshly removed from the agar medium and washed in saline were used. The other organisms were killed by heating to 60° for an hour.

Description of the Cutaneous Lesions

The reaction which followed the intracutaneous inoculations was of the so called delayed type. It began a few hours after the injection as a localized erythema which spread for 12 to 36 hours and then receded. The rapidity of its development was not always an index of its maximal intensity. Swelling of the skin began soon after the erythema and spread peripherally. In severe reactions edema of the subcutaneous tissues occurred, and in the most severe, necrosis of the skin, sometimes preceded by the formation of a small pustule at the site of the injection. The subcutaneous edema sometimes spread ventrally beyond the erythema (presumably by gravity) and persisted after the redness had disappeared.

By the end of 36 hours most of the lesions had begun to recede, but some did not attain their maximum size and intensity until this time.

In our earlier experiments the lesions were observed every few hours for the first 2 days and once a day thereafter for a fortnight. This routine was abandoned when it was found to yield no more information than daily readings for 3 or 4 days and biweekly readings thereafter.

Observation consisted of noting and recording two diameters (the maximum one and that at right angles to it) of the areas of redness and of swelling and the estimated depth of the latter; pustule formation; necrosis or healing.

At the onset of this study it was hoped that the state of hypersensitiveness might be demonstrated by the intracutaneous injection of a dose of organisms (or of a filtrate of liquid culture) which would evoke a reaction in the sensitized but not in the control animal; and a number of preliminary experiments were made with this end in view. It was found, however, to be impracticable in the case of gonococci and meningococci, with the rabbit as the experimental animal, for an inoculum which would regularly give rise to a positive reaction in a sensitized animal, produced one also—though a much smaller one—in the controls. This method was therefore abandoned in favor of the one employed in all of the experiments herein reported; namely, the injection of an inoculum which was known to produce lesions in the controls as well as in the rabbits containing agar foci. The criterion of the sensitization was therefore the relative intensity of the reactions in both groups to an equal dose of the same inoculum. This necessitated the injection of controls every time tests were made, and in the analysis of our data, attention to comparative differences between rather than to absolute sizes of lesions.

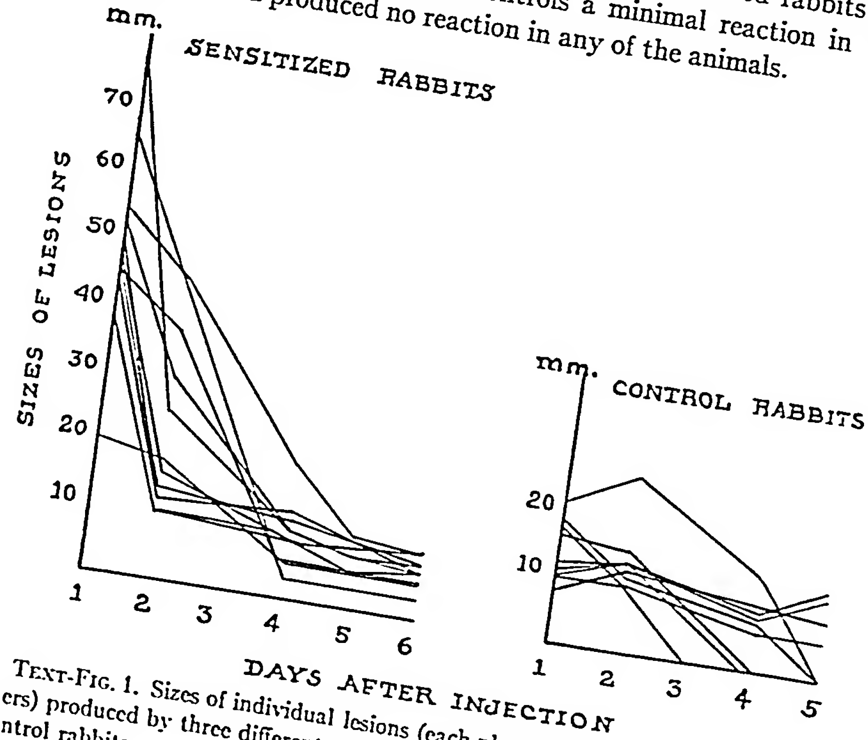
"Secondary Reactions."—Among our control rabbits the "secondary reaction" described by Andrewes, Derick, and Swift (7) was observed but a very few times. In those few instances it occurred during the 2nd week and lasted only a day or two. It consisted merely of a temporary increase in the depth of color and the extent of the fading erythema. With one possible exception, it would have escaped detection but for careful measurement and comparison with data already recorded. In that one instance only was it pronounced enough to have drawn attention to itself.

Development of Cutaneous Hypersensitiveness to Gonococci

Experiment 1.—A subcutaneous agar focus containing viable gonococci was implanted into each of four adult rabbits. Three of them contained gonococcus Strain 1, the other Strain M_6B_2. On the 18th day they, as well as three control rabbits, were tested by intracutaneous inoculation with:

Gonococcus, Strain 1, grown on solid media.
Gonococcus, Strain M_6B_2, grown on solid media.
Gonococcus, Strain 3, grown in liquid media for 18 hours, removed by centrifugation, and made up to the same concentration as the two preceding.
A Berkefeld filtrate of the supernatant of this 18 hour liquid culture of Strain 3.
Uninoculated agar medium.

Result.—The cutaneous reactions induced by all three strains of gonococci were much larger and appreciably more indurated in the rabbits with agar foci than in the controls. These results are presented graphically in Text-fig. 1, in which are plotted the sums of the diameters of these lesions.[6] The suspensions of all three strains produced lesions of equal intensity (within the limits of error of the method) in all of the sensitized rabbits. The filtrate of the 18 hour liquid culture of Strain 3 produced only a moderate reaction (not plotted in Text-fig. 1) in one out of the four sensitized rabbits and none in the others, and in the controls a minimal reaction in two. The agar media produced no reaction in any of the animals.



TEXT-FIG. 1. Sizes of individual lesions (each plotted as the sum of two diameters) produced by three different strains of gonococci in four sensitized and three control rabbits.

This experiment shows then that the two strains of gonococci cross-reacted; that in a young culture in liquid medium the reacting sub-

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[6] This method of graphic representation is used for the sake of simplicity. It should be noted, however, that redness and swelling were invariably more marked in the positive allergic reactions than in the corresponding controls, the difference being usually more striking in appearance than the comparison of the figures recording the diameters of the lesions.

stance is contained entirely or almost entirely within the bodies of the organisms themselves rather than in the liquid medium surrounding; and that the cutaneous reaction is not produced by some substance in the agar medium which might conceivably adhere to the bodies of the organisms grown upon it.

It was likewise shown in subsequent experiments that in the development of hypersensitiveness the agar used in making the focus plays no rôle since rabbits containing uninoculated agar foci behaved exactly like controls.

Additional experiments similar to Experiment 1 failed to disclose any differences, by this method, among six strains of gonococci.

The Development of Hypersensitiveness by a Variety of Organisms

Experiment 2.—A series of rabbits was prepared by the implantation of agar foci. The focus in each of a pair contained one of the following organisms:

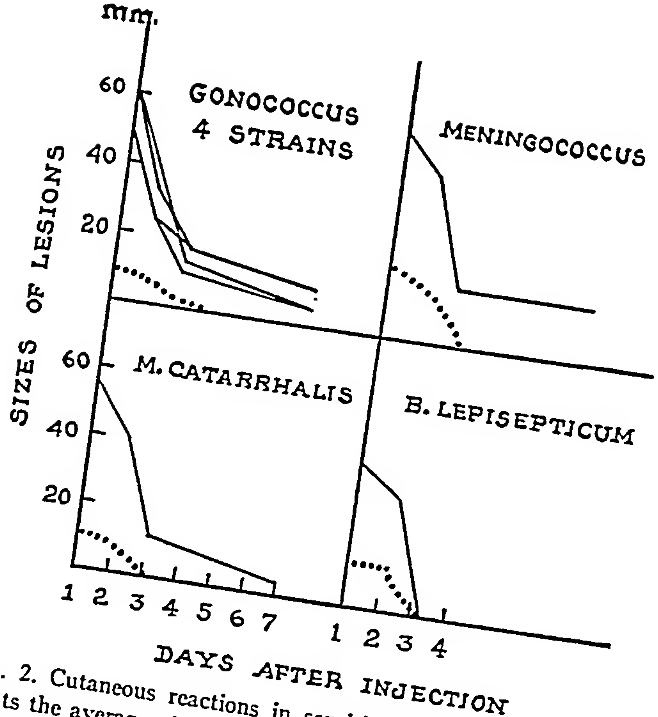
Gonococcus, Strain 1.
Gonococcus, Strain 3.
Gonococcus, Strain 5.
Gonococcus, Strain M_6B_2.
M. catarrhalis.
Meningococcus.
Bact. lepisepticum.

After an interval of 10 days each of these rabbits was injected intracutaneously with its homologous organism. The resulting lesions, plotted in Text-fig. 2, were much larger than those in the control, demonstrating that by this method cutaneous hypersensitiveness could be induced by each of these organisms.

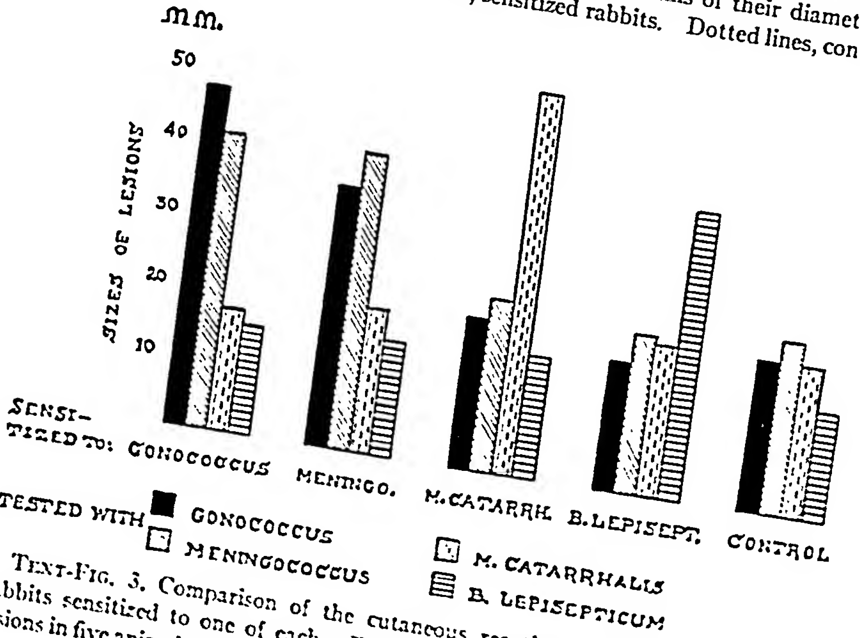
The Specificity of the Cutaneous Reactions

Experiment 3.—Four groups of five rabbits each were prepared by the implantation of agar foci, containing in the case of each group gonococci, meningococci, *M. catarrhalis*, and *Bact. lepisepticum*, respectively. 10 days later each animal, as well as each of five controls, was inoculated intracutaneously with 0.1 cc. of a standard suspension of each of the four organisms mentioned.

Result.—As all of the reactions were maximal at the end of 24 hours, the readings made at that time are plotted in Text-fig. 3. It will be seen that the foci containing meningococci evoked almost as great a sensitivity to gonococci as to meningococci, and *vice versa*, but that cross-reactions in the case of *Micrococcus catarrhalis* and *Bacterium lepisepticum* did not occur. In several other experiments, however,



TEXT-FIG. 2. Cutaneous reactions in sensitized and control rabbits. Each line represents the average of the sizes (plotted as the sums of their diameters) of the lesions in two rabbits. Solid lines, sensitized rabbits. Dotted lines, control rabbits.

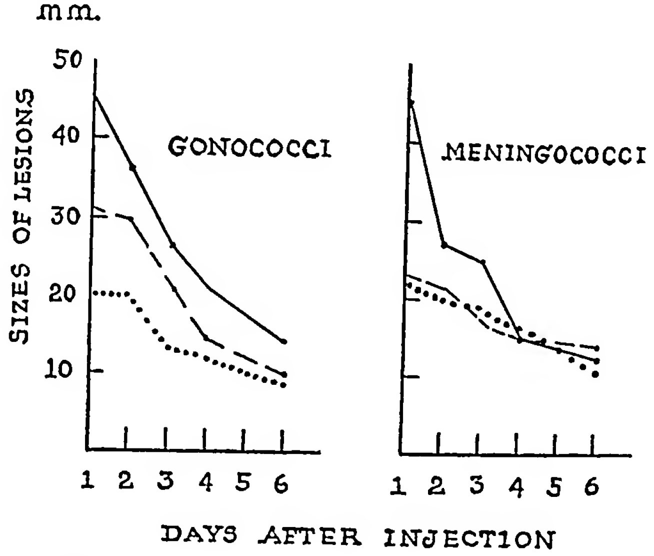


TEXT-FIG. 3. Comparison of the cutaneous reactions to four organisms in rabbits sensitized to one of each. Each column represents the average of the sizes of the lesions in five animals at the end of 24 hours.

M. catarrhalis was found to cross-react to a certain degree with gonococcus and meningococcus.

The Influence of the Size of the Focal Inoculum on the Development of Cutaneous Hypersensitiveness in the Case of Gonococcus

Experiment 4.—Two groups of four and three rabbits respectively were injected with agar foci. The foci in the animals of the former group (four rabbits) contained 1 cc. of a 1:10 suspension of gonococci; in the latter group, 2 cc. of the same suspension. After an interval of 10 days all the rabbits, as well as three normal controls, were injected intracutaneously with standard suspensions of gonococci and meningococci.



TEXT-FIG. 4. Differences in degree of hypersensitiveness effected by focal inocula of different quantities. Solid lines, average sizes of lesions in four rabbits bearing agar foci containing 1 cc. of 1:10 suspension of gonococci. Broken lines, average sizes of lesions in three rabbits bearing agar foci containing 2 cc. of 1:10 suspension of gonococci. Dotted lines, average sizes of lesions in three control rabbits.

Result.—As shown in Text-fig. 4, the cutaneous reactions were greater in the animals which had been prepared by the smaller focal inoculum (1 cc.) than in those which had received the larger focal inoculum (2 cc.) or in the controls. The meningococcal reactions of the second group differed not at all in size from those of the controls, but only in their somewhat deeper color and greater swelling.

Experiment 5.—Two groups of three rabbits each were injected with agar foci containing respectively 2.5 and 5 cc. of a 1:10 suspension of gonococci. After an interval of 10 days these rabbits, as well as two normal controls, were injected intracutaneously with standard suspensions of gonococci and meningococci.

Result.—The result was comparable to that in Experiment 4 in that there was a marked difference (more marked, in fact, than in Experiment 4) between the lesions produced in the two groups by both gonococci and meningococci. In this instance also the animals prepared by the smaller focal inoculum developed the larger cutaneous lesions. The control rabbits developed lesions of the same size, though not quite so indurated as those which had received the focal inoculum of 5 cc.

These two experiments showed clearly that the degree of hypersensitiveness was related to the size of the focal inoculum. The obvious discrepancy between the two experiments lay that in the fact that in Experiment 5, 2.5 cc. of the bacterial suspension sensitized the rabbits very well, whereas in Experiment 4, 2 cc. was much less effective than 1 cc. It was at first thought that this discrepancy could be easily explained as failure of centrifugation to pack the organisms as closely in one instance as in the other. (The experiments were performed several days apart.) But subsequent repetitions of these experiments, made in the hope of establishing an optimum sensitizing dose, showed only that very small and very large focal inocula were either ineffective or much less effective than the doses already mentioned, and also that the animals vary considerably in the degree of sensitiveness which they develop. The interval between sensitization and cutaneous inoculation is considered in a subsequent section.

The Duration of the Hypersensitive State Produced by This Method

In the case of the three Gram-negative diplococci studied, it may be said that although rabbits were occasionally found to be hypersensitive 1 week after the implantation of the infected agar focus, the best reactions usually occurred after an interval of 10 days. By the end of the 3rd week the hypersensitive state produced by this method had usually passed. In the following experiment the influence of the size of the focal inoculum was controlled by sensitizing rabbits with agar foci containing three different amounts of gonococci.

Experiment 6.—Three groups of two, three, and three rabbits each were injected with agar foci containing respectively 1, 2.5, and 5 cc. of a 15 per cent suspension of gonococci. At (approximately) weekly intervals these animals as well as two controls (new controls being introduced at each testing) were injected intracutaneously with standard suspensions of gonococci, meningococci, and *M. catarrhalis.*

Result.—The result of this experiment may be briefly summarized as follows: The most intense reactions occurred in the first group on the second testing (15 days after implantation of the focus), and moderate ones on the next (the 22nd day), but by the fourth test (on the 32nd day) the reactions were no greater than those in the controls. In other words, hypersensitiveness had not developed by the 6th day, was maximal on the 14th, and had ceased by the 32nd. The reactions were sharpest to gonococci, less so to meningococci, and least of all to *M. catarrhalis*; *i.e.*, cross-reactions with meningococci were less pronounced in this experiment than was the case in some of the foregoing. The second group of rabbits (containing foci of 2.5 cc.) reacted less strongly but in the same general way as those first described, while those in the third group showed no evidence of hypersensitivity.

It must not be concluded from the foregoing experiments that the hypersensitive state is always passed by the end of the 3rd week, for rabbits have been found to be somewhat allergic 2½ and even 4 months after the implantation of their foci. Such animals, however, had had not more than two preceding cutaneous tests.

Cutaneous Reactions in Rabbits Intravenously Immunized to Gonococci

From time to time small groups of rabbits which had survived repeated intravenous injections of living gonococci were tested by intracutaneous inoculation with gonococci, meningococci, and *M. catarrhalis.* Of a total of seventeen animals so tested, all developed lesions indistinguishable from the appropriate controls.

DISCUSSION

The experiments here reported demonstrate the practicability of rendering rabbits hypersensitive to three common Neisseriae by implanting into their subcutaneous tissues masses of agar containing living organisms. Mention may again be made of the point that our only criterion of hypersensitiveness has been the local response to intracutaneous injection of bacterial suspensions. The hypersensitive state was found to develop about the beginning of the 2nd week, to be maximal on the 10th to 12th day, and to be gone in most instances by the 4th week. This agrees with the findings of Swift and his coworkers in the case of streptococci, of Julianelle and Avery and of MacKenzie and Woo in the case of pneumococci. Exceptions to our generalization were occasionally encountered, but the statement covers the usual observations.

No relationship could be established between the size of the focal inoculum and the rate of development or duration of the allergic state. But the degree of allergy developing was materially affected by the numbers of organisms contained in the agar focus; for very small and very large inocula both failed to elicit the desired effect. It is possible that in the latter instance the hypersensitive state was of unusually short duration and that we chanced to miss it on each trial; but numerous attempts with this point in mind were unsuccessful.

Although in each of the experiments herein described living bacteria were employed to sensitize the animals to gonococci, meningococci and *M. catarrhalis*, a few experiments which were made indicated that heat-killed organisms functioned almost as effectively. It might be noted in passing that agar foci containing nucleoprotein of the gonococcus[7] rendered rabbits even more highly allergic than (approximately) equivalent quantities of living organisms.

In several of our experiments efforts were made to correlate the precipitin titers of rabbits' sera with their cutaneous reactions, but the results were too inconsistent to justify any conclusion. Precipitins began to be demonstrable in the serum about the same time that

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[7] For these preparations of gonococcal nucleoprotein the authors are indebted to Dr. Alden K. Boor.

hypersensitiveness developed, but among a group of rabbits which seemed, by cutaneous tests, to be equally allergic, some yielded sera containing, and some sera not containing, precipitins for the homologous organism.

As regards the specificity of the allergy engendered by the agar focus method our experiments seemed to warrant the following statements. Animals sensitized to gonococci usually but not always reacted equally or nearly so to meningococci; and *vice versa*. Animals sensitized to gonococci reacted appreciably less strongly to *M. catarrhalis* (and *vice versa*) but more strongly to it than to such organisms as streptococci and staphylococci. Among the Gram-negative diplococci, therefore, gonococci and meningococci are, by this criterion, more closely related to each other than to *M. catarrhalis*. This finding parallels the observation of Boor and Miller on the immunological relationships of their nucleoproteins and carbohydrate fractions.

Animals sensitized to *B. lepisepticum* were not allergic to any of the Gram-negative diplococci employed, and *vice versa*. In comparing these observations with those of Hanger it should be pointed out that all of the suspensions of *Bact. lepisepticum* employed in our experiments were heat-killed because we feared the possibility of contaminating our animal quarters with this organism.

The specificity of reaction observed in our experiments was rather surprising in view of certain reports in which this point is considered. The observations of Hanks and Rettger (12), of Stroem (13), and of Hanger (14) have already been cited. In addition should be mentioned Meyer and Christiansen's (18) experimental study of the typhoidin reaction in rabbits, wherein they report non-specific reactions even with extracts of organisms not at all related to the typhoid group. Zinsser and Parker (3) noted that tuberculous guinea pigs reacted in some instances to pneumococcus residue antigen. And Zinsser and Tamiya (19) found skin tests to be specific only when their animals were moderately hypersensitive, and encountered overlapping when a high degree of allergy was present. The methods employed by these authors to engender the hypersensitive state were not the same as ours, and the difference may account for the discrepancy in result.

The failure to demonstrate hypersensitiveness by the intracutaneous injection of an inoculum or saline extract which would cause no reaction in controls can be explained, we believe, on the basis of two facts. One is the inability, in our hands at least, to obtain the same degree of allergy to the Gram-negative diplococci as is possible to certain other antigens. The other is the toxicity of the proteins of the organisms dealt with. In connection with these two points it is appropriate to comment on the rarity of the occurrence of the "secondary rise" observed by Andrewes, Derick, and Swift to follow intracutaneous injections of streptococci. One possible explanation, which has not yet been subjected to experimental verification, is that the cellular reaction which results from the introduction of such toxic proteins alters in some fashion their composition so that they cease to exist as antigenically identical substances.

No evidence was obtained that gonococci "excrete" the substance responsible for the cutaneous reaction, for filtrates of cultures in liquid medium, filtered immediately after the phase of most rapid multiplication, usually evoked no cutaneous reaction, or at most a feeble one. It was from old liquid cultures, in which autolysis had begun, that reactive filtrates were obtained, or from filtrates of cultures lysed by means of dilute alkali.

SUMMARY AND CONCLUSIONS

By means of the reaction to intracutaneous inoculation with bacterial suspensions in amounts of 0.1 cc., bacterial allergy was demonstrated in rabbits into which had been implanted agar foci containing either gonococci, meningococci, *M. catarrhalis*, or *Bact. lepisepticum*. The criterion of hypersensitiveness was the relative size and intensity of reaction evoked by an identical dose in "agar focus" and control rabbits. Rabbits sensitized to gonococci or meningococci usually reacted indistinguishably to either of these organisms, but were less allergic to *M. catarrhalis*. Similarly, animals sensitized to *M. catarrhalis* gave moderate but not maximal responses to the two former organisms. Cross-reactions did not occur between *Bact. lepisepticum* and any of the three Neisseriae. Animals sensitized to the four organisms mentioned reacted no more intensely than did controls to hemolytic streptococci, staphylococci, and rough pneumococci.

The hypersensitive state was found to begin early in the 2nd week and to end usually by the 4th week, being at its height in most instances on the 10th to 12th days. The number of organisms contained in the agar focus determined the success of the sensitization only to this extent, that very small and very large inocula failed to evoke the allergic state.

Rabbits immunized by intravenous injection of live organisms developed cutaneous reactions indistinguishable from those in controls.

The "secondary rise" of Andrewes, Derick, and Swift was rarely observed.

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FURTHER STUDIES ON THE INFLUENCE OF TESTICLE EXTRACT UPON THE EFFECT OF TOXINS, BACTERIA, AND VIRUSES, AND ON THE SHWARTZMAN AND ARTHUS PHENOMENA

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PLATES 23 TO 25

(Received for publication, June 22, 1933)

While there is general agreement on the enhancing effect of testicle extract on infective processes (1), the literature contains conflicting reports on the effect of the testicle factor on the lesions produced by toxins and enzymes. In collaboration with Hoffman (2), we recorded the fact that testicle extract did not influence the type of lesions produced by *B. coli* toxin or trypsin injection in the skin of rabbits, nor did it enhance the action of tetanus toxin in guinea pigs. However, McClean (3) has reported that testicle extract definitely enhances the local lesions produced by intracutaneous injection of diphtheria toxin. More recently Bier (4) has claimed that the Shwartzman phenomenon is intensified if the testicle factor be added to the "preparatory" intracutaneous injection of the toxin.

We have attempted to elucidate these apparently conflicting findings by investigating the effect of varying concentrations of the agents employed in the experiments, as this seemed to be the most likely variable.

Material and Methods

The essential part of the experimental technique consisted in intracutaneous injection of constant amounts of progressive dilutions of the infective or toxic agents mixed with rat or bull testicle extract prepared in the usual way. The resulting lesions were compared with those produced by the same dilutions of the agents mixed with Ringer's solution or water as controls. This procedure was followed in the experiments with bacteria and viruses and in those with horse serum, in testing the Arthus phenomenon. In other tests the marked individual differences in the response of normal or prepared rabbits to injection of bacterial toxins or the comparative benignity of the skin reactions to certain foreign sera made dilution of the agents unnecessary. The areas of the lesions were determined at suitable intervals and recorded in square centimeters.

Effect of Testicle Extract on Lesions Produced by Bacterial Toxins and on the Shwartzman Phenomenon

If a small amount of certain bacterial toxins is injected intracutaneously and 24 hours later an intravenous injection of the same or another toxin is given, a profound reaction takes place in the injected skin area. This so called Shwartzman phenomenon has been studied both as concerns the influence of testicle extract on the initial lesions from the intracutaneous or preparatory injection, and its influence on the secondary phenomenon.

Experiment.—Rabbits were prepared by the intracutaneous injection of 0.25 cc. of filtrate from a 6 day old broth culture of *B. coli* mixed with testicle extract, and control animals received the same amount of toxin diluted with Ringer's solution. After 24 hours the sizes of the lesions were recorded and the animals were then given an intravenous injection of 2 cc. per kilo of body weight of the same toxin. The resulting changes in the skin lesions were recorded after another 8 hours. The results of the two series of observations in the test and control animals are given in Table I.

In order to eliminate the individual variations between animals, each rabbit of the next group received the test inoculation of toxin and testicle extract and also the toxin diluted with Ringer's solution. The results are recorded in Table II.

In analyzing the results it is necessary to separate the effect of testicle extract on lesions produced by the preparatory intracutaneous injections and the action of the same factor on the lesion developing in the same area after the intravenous injection of the bacterial toxin (Shwartzman phenomenon). Nevertheless, the same principle was manifest in both instances. The area of the lesion produced by the toxin with testicle extract and the extent of the Shwartzman phenomenon were undoubtedly larger than in the controls, but what the lesions had gained in extent they had lost in intensity. This was particularly true of the Shwartzman phenomenon, where instead of the well defined and severe edematous and hemorrhagic character, the lesions were flat, diffuse, and mild. In the animals in which the con-

TABLE I

Effect of Testicle Extract on the Lesions Produced by B. coli Toxin and on the Shwartzman Phenomenon (First Group)

Rabbit No.	Preparatory intracutaneous injection			Toxic injection (2 cc. per kilo of body weight)	
	Material injected with 0.25 cc. of bacterial filtrate	Area of lesion after 24 hrs.	Character of the lesions	Area of lesion after 8 hrs.	Character of the lesions
		sq. cm.		*sq. cm.*	
1	0.25 cc. Ringer's solution	6.0	Strongly congestive, clearly defined	10.8	Strongly hemorrhagic and necrotic, clearly defined. Animal sick
2	0.25 " " "	8.0	Strongly congestive, clearly defined	17.6	Strongly hemorrhagic, clearly defined. Animal died in 2 hrs.
3	0.25 " " "	8.9	Strongly congestive, clearly defined	9.9	Strongly hemorrhagic, clearly defined. Animal sick
4	1.00 " " "	4.9	Mildly congestive, clearly defined	6.5	Strongly hemorrhagic, clearly defined
5	1.00 " " "	12.6	Mildly congestive, clearly defined	20.4	" " " "
6	1.00 " " "	17.0	Mildly congestive, clearly defined	?	Negative
7	0.25 " testicle extract	50.0	Erythematous, diffuse, flat	100.0	Strongly hemorrhagic, diffuse, flat. Animal died in 8 hrs.
8	1.00 " " "	38.5	Strongly congestive, diffuse, flat	66.5	Mildly hemorrhagic, diffuse, flat. Animal very sick
9	1.00 " " "	50.0	Faintly erythematous, diffuse, flat	81.0	Mildly hemorrhagic, diffuse, flat. Animal very sick
10	1.00 " " "	36.9	Faintly erythematous, diffuse, flat	16.0	Faintly erythematous, one hemorrhagic spot
11	1.00 " " "	30.0	Faintly erythematous, diffuse, flat	15.0	Faintly erythematous, one hemorrhagic spot

TABLE II

Effect of Testicle Extract on the Lesions Produced by B. coli Toxin and on the Shwartzman Phenomenon (Second Group)

Rabbit No.	Preparatory intracutaneous injection			Toxic injection (2 cc. per kilo of body weight)	
	Material injected with 0.25 cc. of bacterial filtrate	Area of lesions after 24 hrs.	Character of the lesions	Area of lesions after 8 hrs.	Character of the lesions
		sq. cm.		*sq. cm.*	
1	0.25 cc. Ringer's solution	4.9	Congestive, clearly defined	12.6	Moderately hemorrhagic, clearly defined
	0.25 " testicle extract	?	Faintly erythematous, very diffuse	64.4	" " flat, diffuse
2	0.25 " Ringer's solution	3.3	Congestive, clearly defined	4.0	" " clearly defined
	0.25 " testicle extract	?	Almost unnoticeable erythema	9.2	Faintly erythematous, one small hemorrhagic spot
3	0.25 " Ringer's solution	5.3	Congestive, clearly defined	5.6	Congestive, clearly defined
	0.25 " testicle extract	49.0	Faintly erythematous, very diffuse	—	Practically negative reaction
4	0.25 " Ringer's solution	5.7	Erythematous, clearly defined		Animal died 2 hrs. after injection. No reaction
	0.25 " testicle extract	25.0	Erythematous, very diffuse		

trol lesion was mild, the one resulting from the toxin-testicle extract mixture was extremely benign or in some cases completely suppressed.

In an additional experiment carried out on four rabbits the procedure was varied by injecting the testicle extract into the prepared area at the same time as the intravenous toxin injection was given. This provides a similar spreading out of the Shwartzman reaction with a corresponding lessening of the severity, or even suppression of the reaction.

It may be concluded from these three sets of experiments that testicle extract has no true enhancing property on lesions produced by bacterial toxins used or on the subsequent Shwartzman phenomenon.

Effect of Testicle Extract on the Skin Reactions to Foreign Sera

Human serum, injected into the skin of a rabbit, produces a well defined lesion which reaches its maximum reaction after 24 hours and regresses after 2 to 3 days. Horse serum induces much the same type of reaction but of a much milder nature. These reactions afforded an opportunity for testing the effect of testicle extract on the intensity of the skin lesions induced by inanimate material.

Experiment.—Human and horse sera were diluted with equal parts of testicle extract and with Ringer's solution for control. Each rabbit received an intracutaneous injection of each of the sera with testicle extract and with the serum diluted with Ringer's solution. The results after 48 hours are summarized in Table III.

These tests emphasize the results obtained in the first group of experiments. The spreading or diffusion of the injurious agent brought about by the testicle factor reduces the intensity of the reaction; and if this causes ordinarily but a mild lesion it is spread over so large an area as result of the testicle extract that its effects become barely discernible.

Effect of Testicle Extract on the Arthus Phenomenon

As a further test of the effect of spreading, experiments have been made on the Arthus phenomenon.

Experiment.—Rabbits were sensitized by the injection of 5 cc. of horse serum subcutaneously at 6 day intervals until each had received five injections. 13

TABLE III

Effect of Testicle Extract on the Reactions Produced by Intracutaneous Injections of Human and Horse Serum in the Normal Rabbit

Rabbit No.	Material injected	Area of resulting reactions	Character of the reactions
		sq. cm.	
1	1 cc. horse serum plus 1 cc. Ringer's solution	12.9	Congestive, edematous, clearly defined
	1 " " " " 1 " testicle extract	?	Almost unnoticeable erythema
2	0.5 " " " " 0.5 " Ringer's solution	6.2	Congestive, edematous, clearly defined
	0.5 " " " " 0.5 " testicle extract	0.0	Practically no reaction
3	0.2 " " " " 0.2 " Ringer's solution	4.0	Slightly congestive and edematous, clearly defined
	0.2 " " " " 0.2 " testicle extract	?	No reaction
4	1 " human " " 1 " Ringer's solution	18.0	Strongly congestive and edematous, clearly defined
	1 " " " " 1 " testicle extract	?	Faintly erythematous
5	0.5 " " " " 0.5 " Ringer's solution	16.0	Strongly congestive and edematous, clearly defined
	0.5 " " " " 0.5 " testicle extract	0.0	No reaction

days after the last injection each animal was injected intracutaneously on one side with 1 cc. each of increasing dilutions of horse serum mixed with an equal volume of testicle extract. On the other side the same dilutions of horse serum to which an equal volume of Ringer's solution had been added were injected as controls. The records of the lesions as they appeared after 24 hours are given in Table IV. It should be noted that the lesions described as erythematous completely disappeared by the end of 2 days, while the hemorrhagic or necrotic ones healed slowly. The general characters of the reactions are illustrated in Figs. 1 to 4.

It will be seen that the results recorded for the Shwartzman phenomenon are essentially duplicated in the Arthus phenomenon. The more marked the dissemination of the injected material under the influence of the testicle factor the less intense is the severity of the reaction.

The Effect of Testicle Extract on Infective Agents at High Dilutions

The above experiments show that mild lesions produced by inanimate materials are suppressed or rendered barely detectable if they are spread through a large area. It seemed necessary to study more closely the action of testicle extract on infective agents which are known to be definitely enhanced in their infectivity by this extract. The following experiment is designed to test the effect on dilution of the infecting agents.

Experiment.—Three strains of staphylococcus with widely differing degrees of invasiveness for the skin of rabbits were selected for this test. Suspensions of these organisms were diluted progressively with water, so that they represented 1:200 to 1:20,000 of a 24 hour agar culture. 0.5 cc. of each dilution was mixed with 0.5 cc. of testicle extract and injected intracutaneously in one side of the rabbit. On the other side for control the injections were the same except that water was substituted for the extract.

The same procedure was carried out using vaccine virus as the infecting agent. The source of the virus was the standard testicle pulp of neuro virus described in previous publications. The degree of severity of the lesions as they developed during the period after inoculation is indicated by plus signs. In two of the tests here the spreading factor from an invasive strain of staphylococcus was substituted for testicle extract. The results are brought together in Table V, and the types of lesions are shown in Figs. 5 to 10.

TABLE IV

Effect of Testicle Extract on the Lesions Produced by the Intracutaneous Injection of Horse Serum in the Hypersensitive Rabbit (Arthus Phenomenon)

Rabbit No.	Material injected	Area of lesions	Character of the lesions
		sq. cm.	
1	1 cc. undiluted serum plus 1 cc. Ringer's solution	25.1	Strongly hemorrhagic and necrotic, clearly defined
	“ “ “ “ “ testicle extract	120.0	Moderately congestive, diffuse, flat. Central part hemorrhagic and necrotic
2	“ “ “ “ “ Ringer's solution	31.1	Strongly hemorrhagic and necrotic, clearly defined
	“ “ “ “ “ testicle extract	82.8	Moderately congestive, diffuse, flat. A few hemorrhagic spots
3	“ serum diluted 50% “ “ Ringer's solution	16.5	Strongly hemorrhagic and necrotic, clearly defined
	“ “ “ “ “ “ testicle extract	66.8	Erythematous, diffuse, flat
4	“ “ “ “ “ “ Ringer's solution	16.8	Strongly congestive, clearly defined
	“ “ “ “ “ “ testicle extract	70.7	Moderately congestive, diffuse, flat
3	“ “ “ 20% “ “ Ringer's solution	9.1	Strongly hemorrhagic and necrotic, clearly defined
	“ “ “ “ “ “ testicle extract	66.8	Erythematous, diffuse, flat
4	“ “ “ “ “ “ Ringer's solution	12.9	Strongly congestive, clearly defined
	“ “ “ “ “ “ testicle extract	70.0	Erythematous, diffuse, flat
3	“ “ “ 10% “ “ Ringer's solution	8.4	Moderately hemorrhagic and necrotic, clearly defined
	“ “ “ “ “ “ testicle extract	25.1	Faintly erythematous, diffuse, flat
4	“ “ “ “ “ “ Ringer's solution	11.5	Strongly congestive, clearly defined
	“ “ “ “ “ “ testicle extract	70.0	Faintly erythematous, diffuse, flat

1	1 cc. serum diluted 10% plus 1 cc. Ringer's solution " " " " " " testicle extract	16.0 55.4	Moderately hemorrhagic and necrotic, clearly defined Faintly erythematous, diffuse, flat
2	" " " " " " Ringer's solution " " " " " " testicle extract	16.8 42.2	Strongly congestive, clearly defined Faintly erythematous, diffuse, flat
3	" " " 5% " " Ringer's solution " " " " " " testicle extract	7.0 20 0	Strongly hemorrhagic, clearly defined Faintly erythematous, diffuse, flat
4	" " " " " " Ringer's solution " " " " " " testicle extract	10 8 27.0	Moderately congestive, clearly defined Faintly erythematous, diffuse, flat
3	" " " 2 5% " " Ringer's solution " " " " " " testicle extract	6.25 ?	Erythematous, rather diffused Almost unnoticeable erythema
4	" " " " " " Ringer's solution " " " " " " testicle extract	10.8 ?	Erythematous, clearly defined Almost unnoticeable erythema

factor. Whether this represents a difference in behavior of viruses and bacteria in general, or merely a difference in the effective range of dilution, is being investigated.

It seems probable from these investigations that the reported enhancement by testicle extract of lesions induced by inanimate injurious agents is not in reality a true augmentation of the intensity of the reaction. While the sizes of the lesions under the influence of the spreading factor are undoubtedly larger, what has been gained in size has been lost in intensity. As far as our experiments go, true enhancement by the spreading factor is obtained only in the case of infectious agents.

SUMMARY

The lesions produced by the Shwartzman and Arthus phenomena, as well as those produced by bacterial toxin and foreign sera in the normal rabbit, are spread by testicle extract over a larger area than would normally take place. With this spreading of the lesions there is a definite reduction in their intensity.

Lesions produced by invasive strains of staphylococcus at high dilutions or non-invasive staphylococci at moderate dilutions are definitely lessened in severity or even suppressed by the spreading action of testicle extract. Virus lesions are consistently enhanced by the spreading factor regardless of the dilution.

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EXPLANATION OF PLATES

PLATE 23

FIG. 1. Rabbit 1 (right side). *a*, Arthus phenomenon lesion produced in the hypersensitive animal by the intracutaneous injection of 1 cc. of horse serum plus 1 cc. of Ringer's solution. 24 hours after injection. *b*, lesion produced by 0.1 cc. of horse serum plus 1.9 cc. of Ringer's solution. 24 hours after injection.

scar from the lesion produced by the intracutaneous injection of 1 cc. of horse serum 13 days before.

Fig. 2. Rabbit 1 (left side). *a*, lesion produced by the intracutaneous injection of 1 cc. of horse serum plus 1 cc. of bull testicle extract. 24 hours after injection. *b*, lesion produced by 0.1 cc. of horse serum plus 0.9 cc. of Ringer's solution plus 1 cc. of bull testicle extract. 24 hours after injection.

Fig. 3. Rabbit 2 (right side). *a*, *b*, *c*, *d*, *e*, decreasing Arthus phenomenon lesions produced by the intracutaneous injection in the hypersensitive animal of 0.5, 0.2, 0.1, 0.05, and 0.025 cc. respectively of horse serum diluted with Ringer's solution to a volume of 2 cc. 24 hours after injection. *x*, scar from the lesion produced by the intracutaneous injection of 1 cc. of horse serum 13 days before.

Fig. 4. Rabbit 2 (left side). *a*, *b*, *c*, *d*, *e*, effects produced by the same decreasing amounts of horse serum as in the other side, plus 1 cc. of bull testicle extract and Ringer's solution to a volume of 2 cc. 24 hours after injection.

Plate 24

Fig. 5. Rabbit 3 (left side). *a*, *b*, *c*, *d*, *e*, decreasing lesions produced by the intracutaneous injection of 1:200, 1:1000, 1:2000, 1:10,000, and 1:20,000 respectively of a 24 hour agar culture of a non-invasive strain of staphylococcus, each injection consisting of 0.5 cc. of the bacterial suspension plus 0.5 cc. of water. 24 hours after injection.

Fig. 6. Rabbit 3 (right side). *a*, *b*, *c*, *d*, *e*, effect produced by the same decreasing amounts of non-invasive staphylococcus plus 0.5 cc. of bull testicle extract. Notice the absence of lesions in spots *d* and *e* and the mild lesions in the other spots. 24 hours after injection.

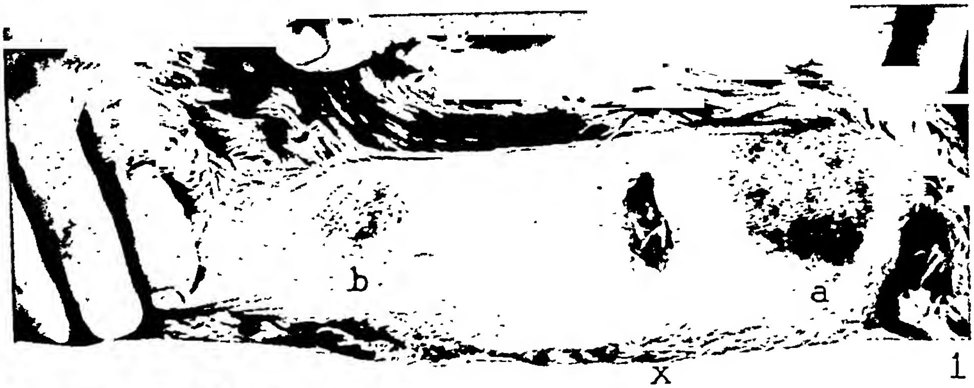
Fig. 7. Rabbit 4 (right side). *a*, *b*, *c*, *d*, *e*, decreasing lesions produced by the intracutaneous injection of 1:200, 1:1000, 1:2000, 1:10,000, and 1:20,000 of a 24 hour agar culture of a very invasive strain of staphylococcus, each injection consisting of 0.5 cc. of bacterial suspension plus 0.5 cc. of water. 24 hours after injection.

Fig. 8. Rabbit 4 (left side). *a*, *b*, *c*, *d*, *e*, lesions produced by the same decreasing amounts of the very invasive staphylococcus plus 0.5 cc. of bull testicle extract. Notice the still slight enhancement in the spots *a* and *b* and the marked inhibition in the other spots. 24 hours after injection.

Plate 25

Fig. 9. Rabbit 5 (left side). *a*, *b*, *c*, *d*, *e*, decreasing lesions produced by the intracutaneous injection of 1:200, 1:1000, 1:2000, 1:10,000, and 1:20,000 of a standard vaccine virus pulp, each injection consisting of 0.5 cc. of the virus suspension plus 0.5 cc. of Ringer's solution. 5 days after inoculation.

Fig. 10. Rabbit 5 (right side). *a*, *b*, *c*, *d*, *e*, lesions produced by the same decreasing amounts of vaccine virus plus 0.5 cc. of bull testicle extract. 5 days after injection. Notice the marked enhancement of the lesions at all dilutions of the virus.



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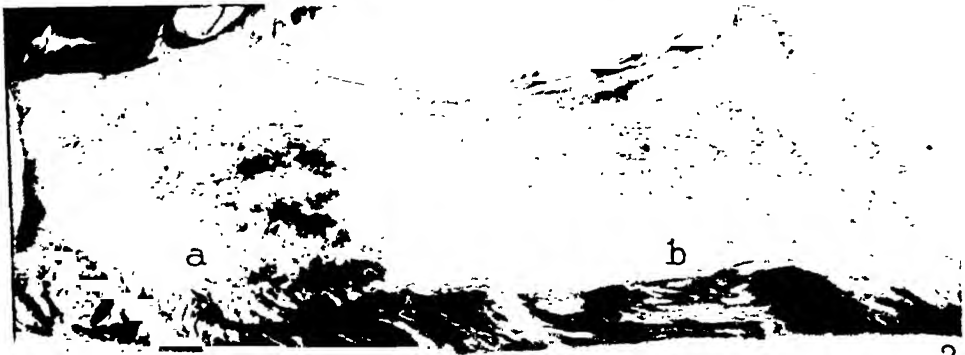

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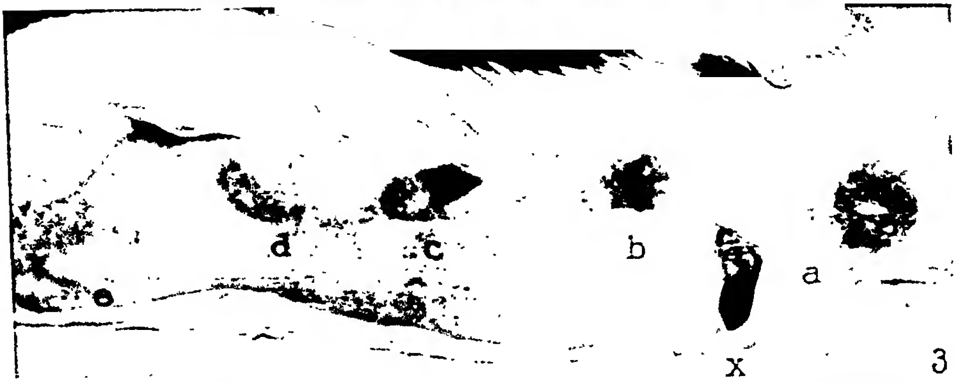

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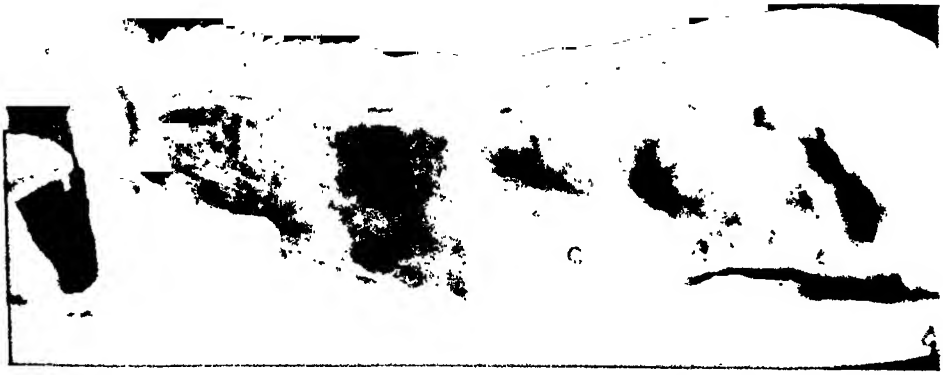

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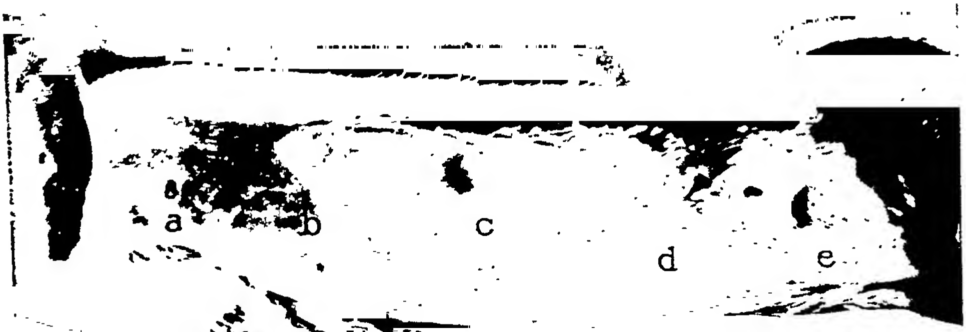

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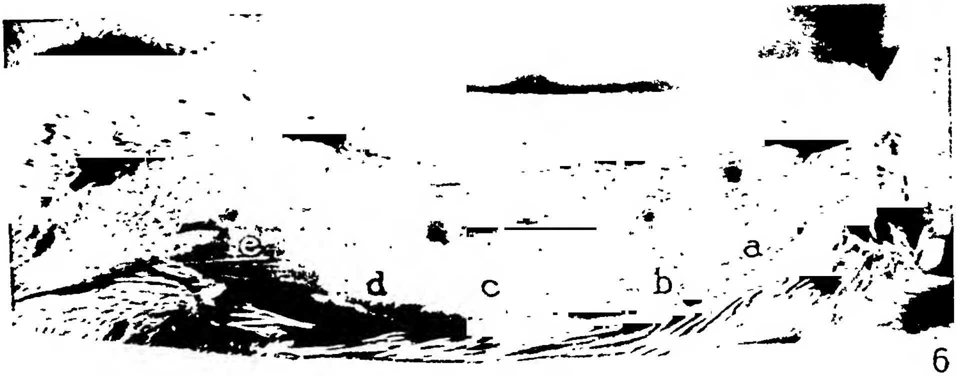

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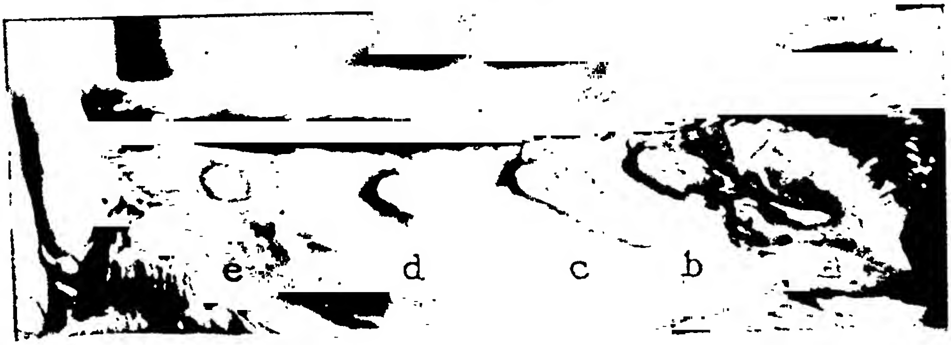

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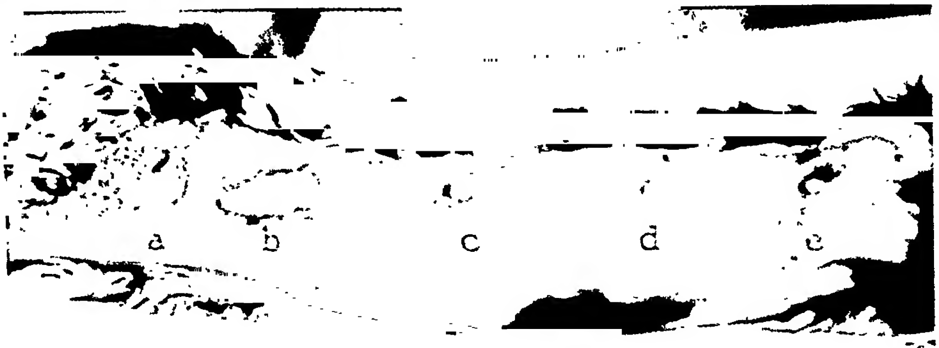

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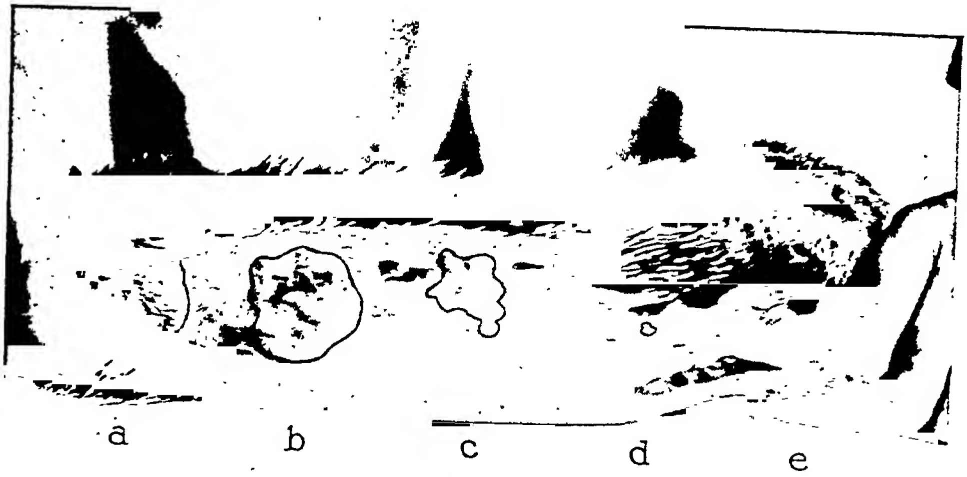

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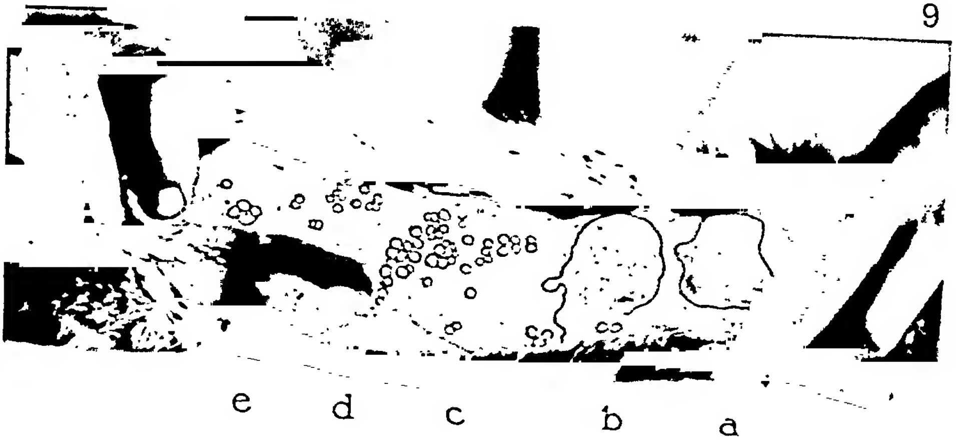

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INTRANASAL VIRULENCE OF PNEUMOCOCCI FOR MICE

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It is generally agreed that pneumococci associated with severe infections of human beings, for example lobar pneumonia, are for the most part antigenically specific and stable (1–6), although rough variants have been reported to occur in the sputum and lung puncture material in some cases of pneumonia (7, 8). Little was known, however, of the stability of pneumococci obtained from healthy persons prior to the observations on small groups of individuals carried out with the aid of Cooper's sera in this laboratory (9, 10). Here it was found that successive strains of pneumococci obtained from a given carrier were for the most part serologically specific, similar and stable in virulence and other characteristics in so far as could be determined in the host. These studies on small groups, confirmed later by Gundel's observations on somewhat larger numbers of persons (11) led us to the view that most strains of pneumococci behave in nature as biological entities with different specific characters which in man are relatively unchanging; but that they do differ among themselves in inherent capacity to incite natural disease, persist in tissues at the normal portal of entry, and spread from host to host (9).

Further evidence on these points has been sought by direct experiments; that is, by intranasal titration of pneumococci in mice.

This procedure, foreshadowed by Stillman's method of injecting mice with ethyl alcohol and spraying them with suspensions of pneumococci (12), and Lange's observations on mice given inhalations of pneumococci (13), developed out of previous experience in this laboratory with intranasal infection of mice with Friedländer-like bacilli (14). Recently Neufeld and Etinger-Tulczynska (15) and Brunzema (16) have reported that undiluted cultures of pneumococci instilled into the nostrils of healthy mice brought about septicemia and death in a few cases.

One advantage can be claimed for the intranasal method of pneumococcus infection in mice—that it employs what is presumably the normal atrium of infection of pneumococci into the host, the upper respiratory tract, and elicits many of the epidemiological features of the natural infection in man. Nevertheless, since the mouse is at best an unnatural host for the pneumococcus, experimental findings with this technique are subject to a limited interpretation.

Experiments dealing with the intranasal virulence for mice of pneumococci freshly isolated from man are described in the present paper.

Technique

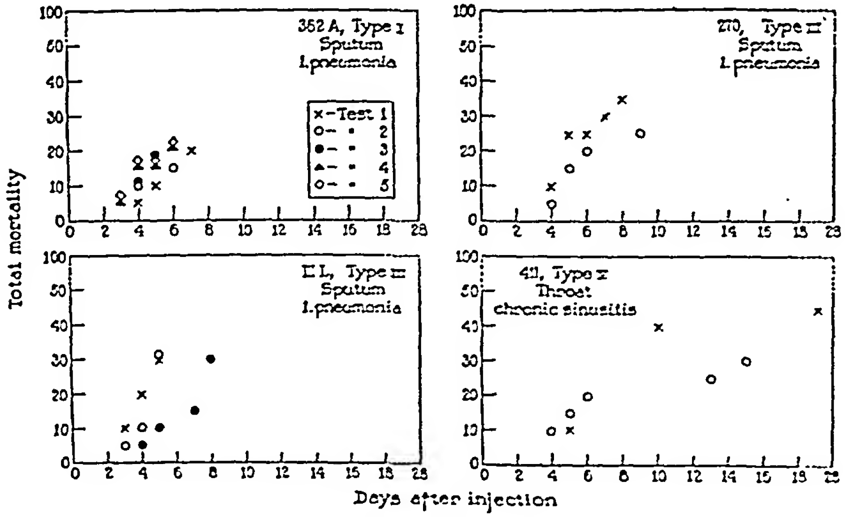
Rockefeller Institute albino mice, free of intercurrent infection, aged 3 months, weighing 16 to 18 gm. each, kept in a special breeding room, and maintained on a diet of bread, milk, and dog biscuit, were used in these experiments. Pneumococci for testing were obtained from sputum or nasopharynx of cases of lobar pneumonia and from the spinal fluid of cases of meningitis usually within 48 hours of admission to the hospital. Material was also obtained from the nasopharynx of healthy persons and spread over the surface of blood agar plates. The sputum or blood broth growth from the nasopharynx culture plates was injected intraperitoneally into mice, and pneumococci obtained later from the heart's blood in pure culture for testing by seeding into pneumococcus broth. Intranasal and intraperitoneal titrations were run on fresh cultures, usually within 72 hours after isolation. For the intranasal test a standard dilution of 1:100 of an 18 hour pneumococcus broth culture was used and administered in 0.03 cc. volume to each mouse, approximately 150,000 organisms. The material was placed at the orifices of the nasal passages of each individual through a 1 cc. tuberculin syringe and blunt needle. The twenty treated animals generally employed for each test were then placed in individual glass jars to prevent reinfection from contact. Animals found dead were autopsied and cultured for the presence of pneumococci. Suitable colonies grown on rabbit blood agar plates were tested in bile and type sera kindly supplied by Miss Cooper (6). Results were expressed as per cent dying of the infection within 14 days. For the intraperitoneal test, dilutions of an 18 hour broth culture from 10^{-1} to 10^{-7} were prepared and given in 1 cc. volumes to mice as follows: 10^{-1} to 10^{-5} each to one mouse, 10^{-6} and 10^{-7} each to two mice. Results were expressed in terms of the exponent of dilution of culture fatal to mice within 4 days.

The presence of pneumococci in the nasal passages of mice was detected by instilling physiological saline into the nares, waiting until it was aspirated and ejected, and then spreading it over the surface of freshly prepared 5 per cent rabbit blood agar plates. The plates were examined after 24 hours' incubation for the presence of pneumococcus-like colonies. These were then selected and identified serologically.

Tests of the ability of pneumococci to spread from experimentally infected individuals to contacts were made by placing ten mice infected intranasally into a single cage 25 x 18 x 12 cm. in dimensions and adding ten normal mice 6 to 48 hours later, and one or two additional normal mice each day thereafter. Carrier tests were made on the entire population at frequent intervals, and autopsies and bacteriological tests on mice dying during the period of observation.

EXPERIMENTS

Early experiments showed that if a given fresh strain of pneumococcus was administered intranasally to mice of different hereditary and environmental experiences, the percentage mortalities in duplicate and repeated titrations were irregular and unpredictable, and again, if the test culture was a stock transfer from rabbit blood agar or broth to blood broth, the mortalities dropped quickly to zero. If, however, the culture was a growth from the blood of a mouse dead of the infection, and the test mice were kept uniform as far as possible, duplicate titrations and titrations repeated at short intervals gave reliable measurements of certain potencies of pneumococci in mice.

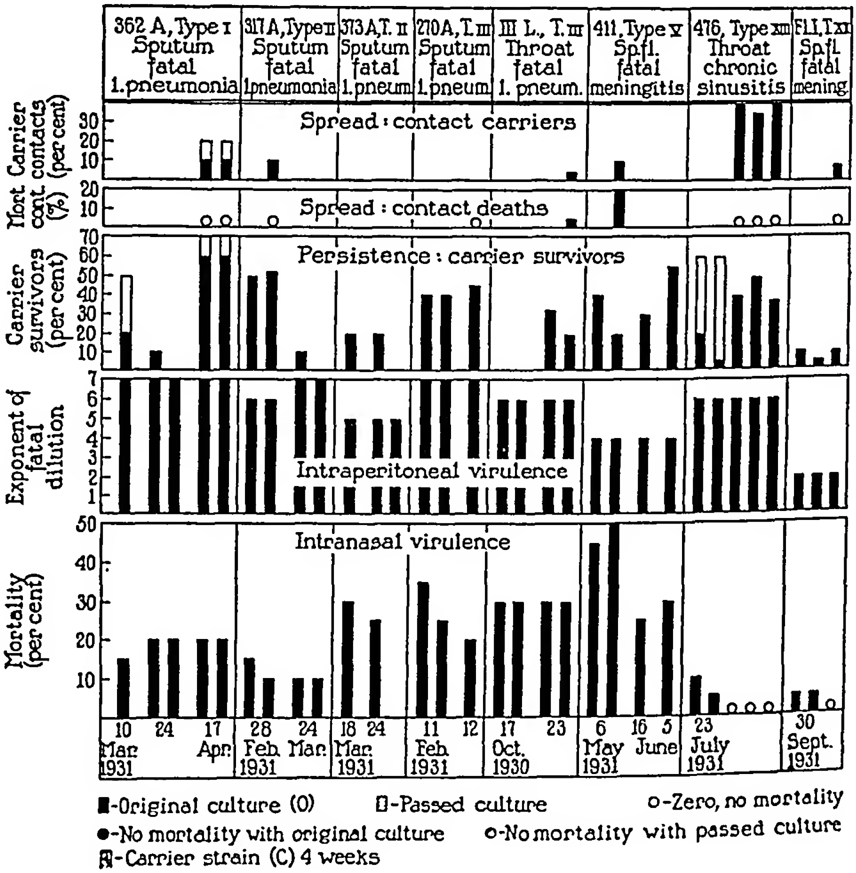


TEXT-FIG. 1

Experiment 1.—Tests of intranasal and intraperitoneal virulence, carrier rates in survivors, and ability to set up the carrier state in contacts or to kill them have been made on 24 fresh strains. Protocols of eight are set forth briefly in Text-figs. 1 and 2.

Four strains described were from the sputum and one from the throat of fatal cases of lobar pneumonia; two were from the spinal fluid of fatal cases of meningitis; one was from the throat of a case of chronic sinusitis. One was Type I; two Type II; two Type III; one Type V; one Type XIII; one Type XX.

Each strain, when placed at the nares of twenty mice, was capable of inducing in some a fatal respiratory tract and general infection. After an incubation period of 2 days, a percentage of mice became ruffled, dyspneic, lost appetite and normal activity, and after 3 to 7 days succumbed to the infection (Text-fig. 1). Blood cultures became positive in fatal cases within 24 hours and contained increasing numbers of pneumococci until death. Some strains persisted in the nasal passages of survivors for at least 4 weeks. The pathology of the infection was septicemia, pneumonia, pleurisy, empyema, pericarditis, and cervical lymphadenitis.[1]



TEXT-FIG. 2

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[1] A detailed report of gross and histological changes will be given in the near future.

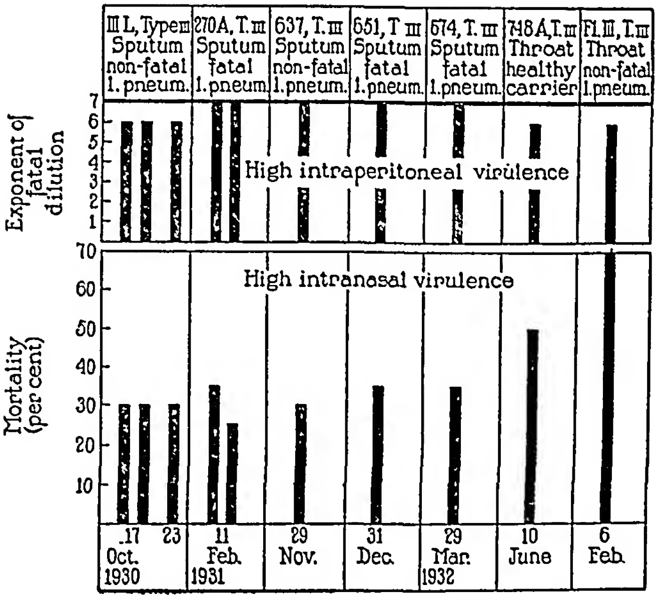
The findings leave no doubt that pneumococci are able by themselves, without the aid of any other microorganism or virus agent, unless some unrecognized form is present in the test mice or cultures, to incite in mice, under stated conditions, certain phenomena of pneumococcus infection.

The titration results indicated (Text-fig. 2), moreover, that a satisfactory control of variables had been achieved, since intranasal tests run in duplicate or repeated at short intervals, resulted usually in mortalities which approximated each other within 10 per cent. An exception is shown in the protocol of Strain 411, in which the mortalities over a 2 weeks period differed by 20 per cent. In these instances the routine method for preserving the virulence of the culture probably did not suffice.

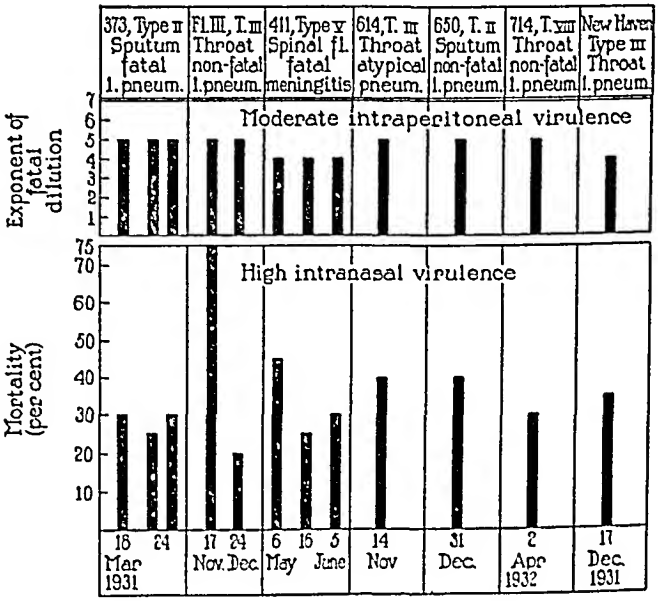
The results indicate also that individual mice of a given test batch differ profoundly in their response to pneumococci. Although all were reared under similar conditions, without previous exposure, and were given the same dose of the same culture at the same time under circumstances in which all known variables were controlled, some proved refractory; some survived and became nasal carriers; others developed cervical adenitis which was usually fatal; others, fatal pneumonia with empyema and pericarditis; and finally, others, fatal septicemia and death within 72 hours. These differences and others brought out in histopathological studies to be described later were apparent even when the undiluted culture was employed and are ascribable not to chance but to individual differences in inherent resistance factors.

The tests brought out a parallelism between degree of intranasal virulence and tendency to persist in survivors and spread to contacts to the extent that strains which spread to contacts were invariably of high intranasal virulence and strains which set up the carrier state in survivors were generally of moderate or high intranasal virulence.

Finally, the strains displayed individualities with respect to their intranasal virulences and abilities to persist and spread to contacts. Some exhibited high intranasal and low intraperitoneal virulences—No. 411,—others, low intranasal and high intraperitoneal virulences—No. 317A,—while still others showed intranasal and intraperitoneal virulences running parallel—No. 270A. Some, but by no means all



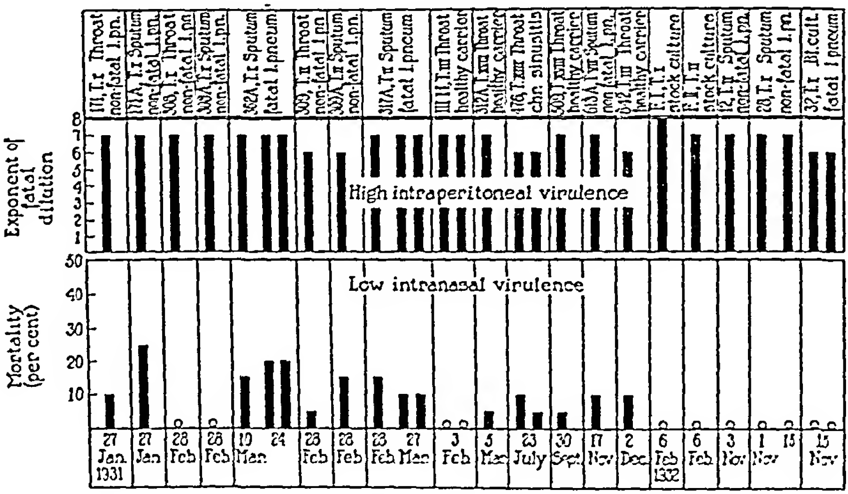
TEXT-FIG. 3*a*



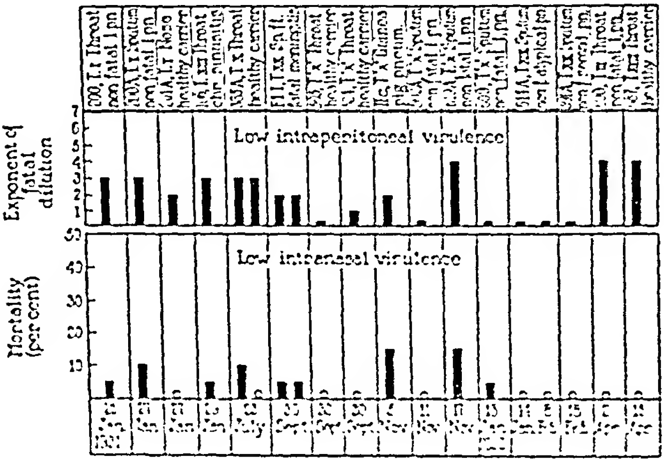
TEXT-FIG. 3*b*

strains spread from infected to contact mice, inciting in them in turn the carrier state or fatal infection.

The individualities of pneumococci with respect to infective potencies in mice brought out in the previous experiment were analyzed



TEXT-FIG. 3c



TEXT-FIG. 3d

further by comparing intranasal and intraperitoneal virulences of the twenty-four strains discussed above and an additional twenty-six strains.

Experiment 2.—Of the total 50 strains, eleven were Type I, ten from cases and one from a healthy carrier; seven were Type II from cases; fourteen were Type III, eleven from cases and three from carriers; eighteen were other named and unnamed types, nine from cases and nine from carriers. As stated before, strains from cases were obtained usually within 48 hours of admission to the hospital and strains from carriers directly from the throats of healthy persons. All strains were of the so called "smooth colony" variety. Simultaneous intranasal and intraperitoneal tests were run with the same culture on twenty and ten mice respectively, as described above, sometimes in duplicate, sometimes at short or occasionally long intervals.

The results of 69 intranasal titrations on a total of 1,380 mice and 69 simultaneous intraperitoneal titrations of 690 mice on the 50 strains summarized in Text-fig. 3 bear on three main points. First, there were repeated demonstrations of the ability of certain strains of pneumococci to incite a characteristic picture of a natural infection. Second, there was additional evidence of the individuality of strains with respect to intranasal virulence. Eight were classed as high in intranasal, 38.3 ± 3.8 (standard error) per cent and high in intraperitoneal, 6.5 ± 0.08 virulences; seven as high in intranasal, 31.2 ± 2.2 per cent, and moderate in intraperitoneal, 4.7 ± 0.07 virulences; nineteen as low in intranasal, 7.4 ± 1.5 per cent, and high in intraperitoneal, 6.8 ± 0.03 virulences; and sixteen as low in intranasal, 3.9 ± 1.2 per cent, and low in intraperitoneal, 1.8 ± 0.34 per cent virulences. One exceptional strain, No. 171 A, with high intraperitoneal virulence, might be placed either in the high or low intranasal groups. It was included in the latter with most of the other Type I strains.

TABLE I

Classification of Serological Types of Pneumococci According to Intranasal and Intraperitoneal Virulence

Type of pneumococcus	No. of strains	Per cent with high intranasal, high intraperitoneal virulence	Per cent with high intranasal, moderate intraperitoneal virulence	Per cent with low intranasal, high intraperitoneal virulence	Per cent with low intranasal, low intraperitoneal virulence
I	11	—	—	72.7	27.3
II	7	—	28.5	71.5	—
III	14	57.1	21.4	14.3	7.2
Others	18	—	11.1	22.2	66.6

Third, there were positive and negative relationships between amount of intranasal virulence of strains and other properties. Thus, intranasal virulence did not parallel degree of intraperitoneal virulence in 50 per cent of cases. Moreover, strains of both high and low intranasal virulences formed smooth colonies suggesting lack of complete relationship between intranasal virulence and S substance. Again, degree of intranasal virulence did not appear to be related to source of strain in humans. Of the eleven Type III strains from cases, ten were of high intranasal virulence, while of three Type III strains from the nasopharynx of healthy persons, one was of high intranasal virulence.[2] A possible relationship to serological type is suggested by the data (Table I). Of eleven Type I strains, all were of low intranasal virulence, although 72 per cent were of high intraperitoneal and 27.3 per cent low intraperitoneal virulence. Of seven Type II strains, 28.5 per cent were of high intranasal and moderate intranasal virulence, and 71.5 per cent of low intranasal and high intraperitoneal virulence. Of fourteen Type III strains, 78.5 per cent were of high intranasal virulence—91 per cent of Type III strains from cases belonged in this category. And finally, of eighteen other named and unnamed strains, only 11.1 per cent showed high intranasal virulences. In brief, Type I strains were of low intranasal virulence; Type II strains low or high; Type III strains high; and strains of other types were generally low. The possible significance of these relationships will be discussed later.

Titrations of sputum and throat strains from the same person and different strains of the same type obtained at the same time from the same person gave comparable results.

The remainder of this report deals with the stability of intranasal virulence of pneumococci for mice. Pneumococci are perhaps the classic example of bacteria pathogenic for human beings, whose virulence by animal test is regarded as labile. This view is based on the fact that repeated intraperitoneal passage in mice of a laboratory Neufeld Type I strain, the pathogenicity of which has dropped so low that 100,000,000 organisms injected intraperitoneally fail to kill, will in certain cases have the consequence that as few as ten organisms prove

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[2] Eighteen additional carrier Type III strains obtained from sixteen young adults over a 5 weeks period did not differ significantly in intranasal virulence from case Type III strains isolated and titrated at about the same time.

fatal by the intraperitoneal route. These and similar experiments with streptococci and other organisms have been given broad epidemiological interpretation. Opposed to these data, however, are experiments with bacteria native to the test host in which freshly isolated strains are administered by way of the normal portal of entry in doses comparable with those in nature. Under these conditions, the manifestations of the natural infections are observed under controlled circumstances and virulence has proved relatively stable (17).

The opposing views have now been tested by comparing the stability of what might be called relatively natural, that is intranasal virulence, and artificial or intraperitoneal virulence of fresh strains of pneumococci. The test is incomplete in that the test animal, the mouse, is essentially a foreign host. To this extent, therefore, not only the intraperitoneal test, but the intranasal test as well, must be regarded as unnatural.

The first series of experiments dealt with the effect of the passage of pneumococci from host to host by way of the nose on both intranasal and intraperitoneal virulences.

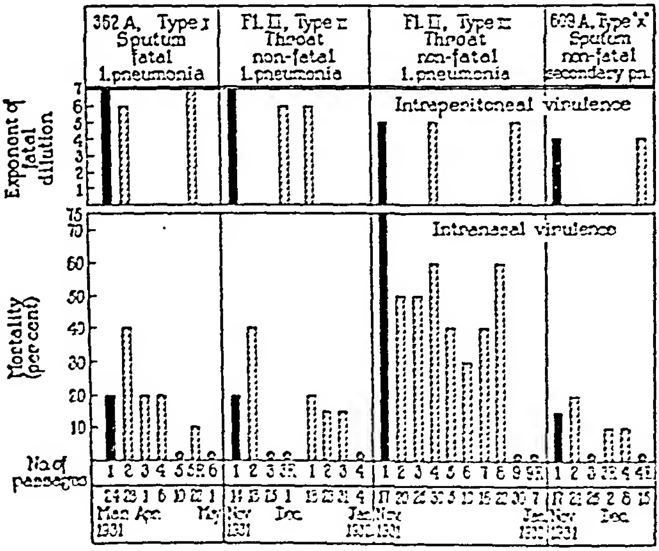
Experiment 3.—A series of passages was made by transferring repeatedly a blood culture from a mouse dying of the infection to the nasal passages of healthy mice.

Nineteen strains were employed; two Type I, five Type II, ten Type III, and two other named types from cases of lobar pneumonia. The strain, first seeded into pneumococcus broth from the blood of a mouse dying after intraperitoneal injection of the organisms, was diluted 1:100 and given in 0.03 cc. quantities intranasally to ten or twenty mice according to the usual routine. From the heart's blood of the first mouse to die following the intranasal instillation, a culture was taken, seeded to broth, diluted, and given intranasally as before to a second batch of mice. This procedure was repeated routinely. Intraperitoneal virulence tests were done on the unpassed and suitable passed cultures. Carrier tests were made on survivors at various intervals after exposure.

Protocols of tests with four strains are given in Text-fig. 4. Passage led invariably to a sudden drop in intranasal virulence from a level characteristic of the strain to zero. This drop occurred after few or many passages, depending on the strain tested. No change in the intraperitoneal virulence of the passage culture was detected. The smooth type of colony on blood agar and agglutinative properties were apparently unaltered. Persistence of the organisms as measured by carrier rates did not change materially with passage nor with the drop of intranasal virulence to zero.

Experiment 4.—In this series, pneumococci were passed from the nasal passages of surviving carriers to the nasal passages of unexposed healthy individuals.

The following procedure was carried out on four strains; one Type I, one Type II, and two Type III. The test strain was injected into the peritoneal cavity of a mouse and recovered 24 hours later from the dead animal's heart blood. The culture, grown 8 hours in blood broth, was diluted as usual and given intranasally to twenty mice. Approximately 2 weeks later, carrier tests were done on survivors, a pneumococcus colony from a positive plate transferred to broth, incubated 18 hours, diluted, and administered intranasally to ten mice. This procedure was repeated. As a control, case to case passages, as described in Experiment 3, were run simultaneously with the same strain. That is, from the first mouse to die of the first group infected intranasally, a blood culture was obtained and given intranasally to a second group. This was continued in series.

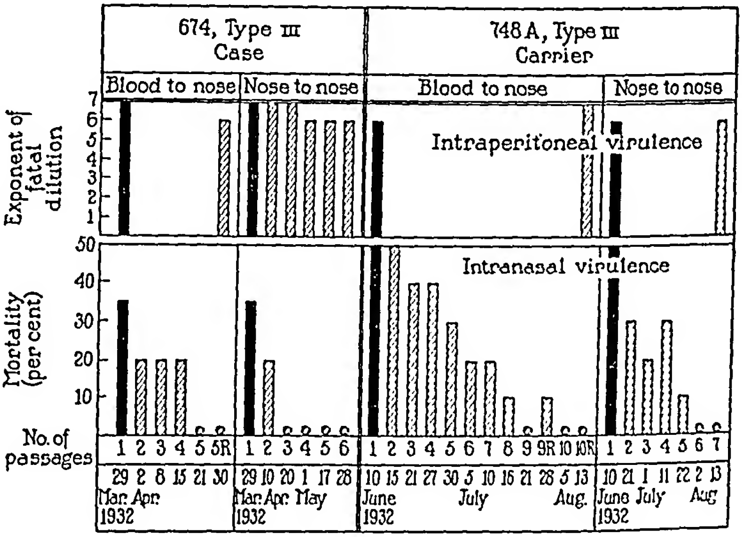


TEXT-FIG. 4

The results of intranasal passage of two strains are shown in Text-fig. 5.

In each instance, passage of pneumococcus from nose to nose resulted in a drop in its intranasal virulence from its characteristic level to zero. Indeed, the drop occurred more quickly when the culture was passed *via* this relatively normal route than when it was passed from blood of fatal cases to nasal passages of healthy mice. Intraperitoneal virulence, however, was not altered. Persistence, as measured by carrier rates, was apparently not affected. Smooth type colony and agglutinative characteristics remained apparently unchanged.

Experiment 5.—Strains of pneumococci were not only passed intranasally but were tested after a sojourn of several weeks in the nasal passages of an individual.



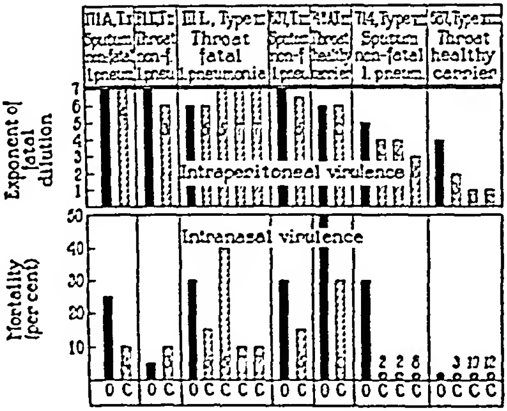
TEXT-FIG. 5

A given strain was instilled into the nasal passages of mice in the routine manner. 2 to 12 weeks later, carrier tests were made on survivors and cultures from positive cases were seeded into blood broth, incubated 18 hours, diluted, and given intranasally to a test group of twenty mice. The results were compared with those of a similar titration made with the freshly isolated strain given the usual single peritoneal passage. Intraperitoneal virulences of carrier strains were also determined and compared with those of the freshly isolated strain.

The results of tests on fourteen carrier cultures from seven strains, one Type I, one Type II, three Type III, and two other named types, are given in Text-fig. 6. In no instance was the intranasal virulence of a carrier culture significantly greater than that of the original strain; on the contrary, it was usually markedly decreased. Intraperitoneal virulence, smooth colony form, agglutinative, and other properties were apparently unaltered.

To summarize briefly, passage of pneumococci through mice by way of the nose led consistently to a loss of intranasal virulence; such passage, however, did not change the intraperitoneal virulence, nor colony and agglutinative properties.

Finally, passages were made by transfers of mouse heart's blood cultures without cultivation on media to peritoneal cavity of mice. Under these conditions certain laboratory strains are known to increase in intraperitoneal virulence.

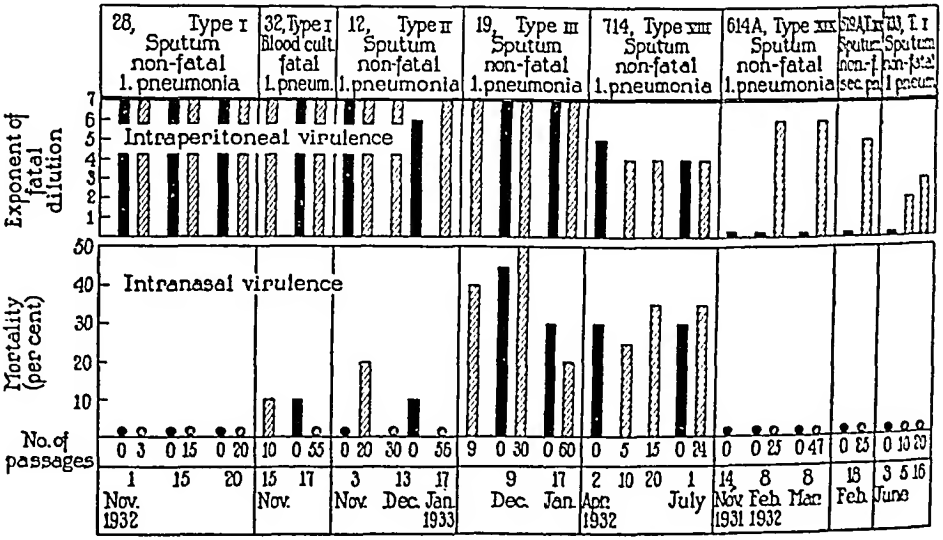


TEXT-FIG. 6

Experiment 6.—Serial intraperitoneal passages were carried out on three Type I strains, one Type II, four Type III, and one each of Types VIII, XIX, XX, and X. These were all smooth colony forms freshly isolated from human cases of lobar pneumonia.

Each test culture was injected into the peritoneum of a mouse and transferred shortly before or after the death of the animal by removing the heart, cutting it into small bits aseptically, and washing it with 2 cc. of pneumococcus broth. This broth containing pneumococci from the heart's blood was then immediately injected in 1 cc. quantities into the peritoneum of two mice. This passage procedure was continued routinely every 24 hours. At suitable intervals comparative titrations were made of passed and unpassed cultures. The passed culture was taken from the heart's blood of a mouse dead following intraperitoneal injection, placed in pneumococcus broth, and grown 18 hours in the incubator; the unpassed culture was taken from the stock blood agar slant, injected into the peritoneum of a mouse, transferred from its heart's blood shortly before or after death to pneumococcus broth and incubated 18 hours. The numbers of organisms per cubic centimeter in the unpassed and passed cultures were found to be similar. Both were diluted in the routine manner and administered intranasally and intraperitoneally into the usual batches of twenty and ten mice respectively.

The results of these tests are summarized in Text-fig. 7. The first four strains, Nos. 28, 32, 12, and 19, were initially high in intraperitoneal virulence. No. 28, Type I, was tested on three occasions by simultaneous titrations of unpassed and passed cultures: Nov. 1, 1932, three passages; Nov. 15, 1932, fifteen passages; Nov. 20, 1932, twenty passages. In each instance the intraperitoneal virulence was maximum, 10^{-7}, and the intranasal virulence zero. Strain 32, Type I, was tested twice: once on Nov. 15, 1932, when the ten passage strain showed an intraperitoneal virulence of 10^{-7} and an intranasal virulence of 10 per cent, and again on Jan. 17, 1933, after 55 passages, when simultaneous titrations of unpassed and passed cultures showed intraperitoneal virulences of 10^{-7} and intranasal virulences of 10 per cent and 0 per cent respectively. Strain 12, Type III, was tested three times: once on Nov. 3, 1932, with simultaneous titrations of unpassed and twenty passage cultures showing intraperitoneal virulences of 10^{-7} and intranasal virulences of 0 per cent and 20 per cent respectively; again on Dec. 13, 1932, when the unpassed culture showed an intraperitoneal virulence of 10^{-6} and an in-



TEXT-FIG. 7

tranasal virulence of 10 per cent, and the thirty passage culture an intraperitoneal virulence of 10^{-7} and intranasal virulence of 0 per cent; and a third time on Jan. 17, 1933, when the 56 passage culture showed an intraperitoneal virulence of 10^{-7} and an intranasal virulence of 0 per cent. Strain 19, Type III, was tested three times,—once on Nov. 1, 1932, when the nine passage culture showed an intraperitoneal virulence of 10^{-7} and an intranasal virulence of 40 per cent; again on Dec. 9, 1932, when the unpassed and thirty passage cultures showed intraperitoneal virulences of 10^{-7} and intranasal virulences of 45 per cent and 50 per cent respectively; and finally on Jan. 17, 1933, when the 60 passage culture showed an intraperitoneal virulence of 10^{-7} and an intranasal virulence of 20 per cent. The other four strains were initially low in intraperitoneal virulence. Strain 714, Type VIII, showed unpassed on Apr. 2, 1932, an intraperitoneal virulence of 10^{-5} and an intranasal virulence of 30 per cent. On June 5, 1932, the five passage culture showed an intraperitoneal virulence of 10^{-4} and an intranasal virulence of 25 per cent; on June 20, 1932, the fifteen passage culture an intraperitoneal virulence of 10^{-4} and intranasal virulence of 35 per cent; and finally, on July 1, 1932, the unpassed and twenty-four passage cultures, an intraperitoneal virulence of 10^{-4} and intranasal virulences of 30 per cent and 35 per cent respectively. Strain 614 A, Type XIX, showed on Nov. 14, 1931, and Feb. 8, 1932, the unpassed culture to be of 1 cc. intraperitoneal and 0 per cent intranasal virulences. On Feb. 8, 1932, a twenty-five passage culture showed an intraperitoneal virulence of 10^{-6} and an intranasal virulence of 0 per cent; on Mar. 8, 1932, simultaneous tests showed the unpassed culture to be of 1 cc. intraperitoneal and 0 per cent intranasal virulences and the forty-seven passage culture of 10^{-5} intraperitoneal and 0 per cent intranasal virulences. Strain 591 A, Type XX, was tested on Feb. 18, 1932, after twenty-five passages. The unpassed culture showed an intraperitoneal virulence of 1 cc. and an intranasal virulence of 0 per cent; the passage culture an intraperitoneal virulence of 10^{-5} and an intranasal virulence of 0 per cent. Strain 733, Type I, showed an intraperitoneal virulence of the unpassed culture on June 3, 1932, of 1 cc. and an intranasal virulence of 0 per cent; on June 5, 1932, the ten passage culture had increased in intraperitoneal virulence to 10^{-2}, but its intranasal virulence remained at 0 per cent; and on June 16, 1932, the twenty passage strain, when injected intraperitoneally, killed in a dilution of 10^{-3}, but failed to kill when given intranasally.

In brief, fresh strains of pneumococci from human cases of initially high or low intraperitoneal virulence, when passed through mice by intraperitoneal injections, did not increase in intranasal virulence but maintained their characteristic level. Some strains did show an increase from low to high intraperitoneal virulence. No changes in colony form or agglutinative properties were noted.

Two fresh strains from healthy carriers gave similar results.

DISCUSSION

The nasal instillations described in this report were made with freshly isolated strains of smooth colony forms of pneumococci from different sources under rigidly standardized conditions, such as are essential in this type of work. The technique reproduced normal conditions in so far as small doses, portal of entry, and setting up of natural infections are concerned, and was artificial in that pneumococci in nature do not ordinarily infect mice. The data bear on special questions of pneumococcus infection and more general questions of epidemiology.

The first question relative to pneumococcus infection is whether the pneumococcus in nature is a primary or secondary invader. The time-honored belief in its essential pathogenicity (18, 19) is being questioned in the light of the realization that pneumococci occur without harm in the nasopharynx of healthy persons, that they do not by themselves readily incite lobar pneumonia in the experimental animal, and that, like influenza bacilli and other organisms, they may give rise to more typical experimental infection when associated with a virus. The present experiments, however, confirming and amplifying previous work (12–16), support the older view in demonstrating that pneumococci are capable by themselves,—unless indeed the action of some attendant virus with unique, unknown properties is hypothesized,—of inciting a natural infection in mice, persisting in the nasopharynx of survivors, and spreading to contacts.

The next point to be noted is that any given strain of pneumococcus evoked different responses in different individual mice and in different batches from known susceptible or resistant stocks (20), ranging from the refractory or carrier state to acute septicemia and death. This series of differences compares closely with those described in native mouse typhoid (21) and rabbit and fowl Pasteurella infections (22, 23) especially, in which all variables save those referable to the host were apparently controlled. The finding agrees with the recent observations of Goodner and Stillman (24) on intracutaneous infections of rabbits with pneumococci, indicating that degree of infection depends as much upon host as bacterial properties (17).

Third, the experiments bear out the observational data on human beings as concerns the individuality of strains of pneumococci of the same or different types (9, 10). Under the conditions specified, strains have shown determinate and characteristic pathogenic properties with no evidence of colony dissociation or serological type transformations.

Fourth, a certain lack of relation between degree of nasal virulence of pneumococci and amount of specific substance present, already commented on by others who have encountered smooth colony strains with no intraperitoneal mouse virulence (25), is suggested by the facts that the intranasal virulence of smooth colony strains may be high or low irrespective of intraperitoneal virulence, and drops to zero when strains are passed through mice by intranasal instillations, without affecting intraperitoneal virulence or smooth colony form.

Fifth, to the extent that intranasal titrations measure the natural virulence of pneumococci for mice, Type III strains must be regarded as more virulent than Type II, and Type II more virulent than Type I; but whether or not intranasal titrations in mice measure the natural virulence of pneumococci for human beings is a matter for conjecture. True, the intranasal method shows in the case of Types I, II, and III strains relative mouse mortalities which are consistent with human pneumonia mortalities. But since mortalities of human cases must reflect uncontrolled host as well as virulence factors (26), there is in reality very little basis for comparison.

Two findings of general epidemiological significance bearing on the question of stability of bacterial virulence are that the intranasal virulence of pneumococci for mice was in no case enhanced, and instead of remaining stable dropped to zero when the organisms were passed by way of the nose. Failure to increase intranasal virulence of pneumococci (even under conditions in which the intraperitoneal virulence was enhanced) is in agreement with experience with other bacteria tested in their native host under relatively natural conditions (17). Failure to maintain the characteristic virulence of pneumococci, the phenomenon of its total loss, is the first exception encountered by us to the general finding that native virulence is relatively stable. It is referable in all probability to the fact that in nature pneumococci do not readily infect and hence that we are not actually dealing with native virulence.

CONCLUSIONS

1. Smooth colony pneumococci fresh from human beings, instilled in small doses into the nasal passages of special mice raised under standard conditions, brought about a characteristic infection and this spread to healthy contacts inciting in them the carrier state or fatal infection.

2. Differences in individual host response to the same dose of a given culture ranged from a complete refractory or nasopharyngeal carrier state, or a local cervical lymphadenitis, to fatal lobular or lobar pneumonias with or without pleurisy, empyema, and pericarditis, and acute fatal septicemia.

3. Pneumococci exhibited consistent individual strain differences with respect to ability to infect, when instilled intranasally into mice, and also differences in the spread to contacts. Degree of intranasal virulence paralleled demonstrable ability to spread to contacts.

4. Degree of intranasal virulence of strains did not parallel intraperitoneal virulence in 50 per cent of strains—high intranasal was accompanied by either high or moderate intraperitoneal virulence, and low intranasal by high, moderate, or low intraperitoneal virulence.

5. Type III strains were of relatively high intranasal and intraperitoneal virulences; Type II strains mostly low in intranasal but high or moderate in intraperitoneal virulence; Type I strains all low in intranasal but either high or moderate in intraperitoneal virulence. Most strains of other types were low both in intranasal and intraperitoneal virulences.

6. The intranasal virulence of pneumococci was not enhanced by animal passage. Nasal passage reduced the intranasal virulence to zero but did not affect intraperitoneal virulence, colony form, and agglutinative specificity. Passage by the intraperitoneal method maintained the characteristic level of intranasal virulence for a period, increased intraperitoneal virulence in some instances, but did not affect colony form or agglutinative properties.

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THE FIBRINOLYTIC ACTIVITY OF HEMOLYTIC STREPTOCOCCI

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The findings presented in this communication demonstrate the capacity of broth cultures of hemolytic streptococci to liquefy rapidly the clotted fibrin of normal human plasma. The experiments have been carried out in such a manner as to emphasize the rapidity with which active cultures transform solid clot into a completely liquid state, and to bring out other special characteristics of the fibrinolysis by hemolytic streptococci which differ in some respects from the orderly digestion of solid protein material by proteolytic enzymes. The observations contained in this report are chiefly limited to a consideration of the presence of fibrinolytic substances in cultures and the conditions of fibrin coagulation which influence the occurrence of liquefaction. Additional lines of investigation, suggested by the results, are not yet complete. Consequently the present communication makes only brief mention of some of the results which will be presented in detail in subsequent publications.

The experimental conditions under which the clot-dissolving property of cultures is most strikingly demonstrable consist in mixing the cultures with plasma or fibrinogen before inducing clot formation. By this procedure the organisms and their products are disseminated within the body of the clot as it forms, thus affording maximum surface contact between the active bacterial agent and the fibrin substrate. Under these conditions the quantity of plasma clot employed in the experiments is liquefied in a few minutes, whereas the same amount of plasma, clotted before the addition of cultures, requires several hours incubation to effect the same degree of dissolution.

The strains of *Streptococcus hemolyticus* employed in the tests have been derived from patients suffering from various manifestations of acute streptococcus infections. The sources of the cultures are given in Table I and the list includes streptococci isolated from cases of septicemia, erysipelas, scarlet fever, acute tonsillitis, cellulitis, and other diseases. In addition to the human strains, hemolytic streptococci of animal origin have also been used in comparable tests. Observations have also been made with strains of other pathogenic bacterial species for the purpose of determining the presence or absence of a similar fibrinolytic property.

Whole plasma from human beings and rabbits, and fibrinogen chemically isolated from the plasma of each species, have served as a source of fibrin. Clot formation was induced by appropriate coagulants which are described in the detailed experiments.

Materials and Methods

Cultures.—NaCl meat infusion broth, adjusted to pH 7.6, and containing 0.05 per cent dextrose, has been the culture medium regularly employed. The broth contains 1 per cent peptone but is not buffered. The final pH, after growth has occurred, rarely goes below 7.2. The tests were performed with fresh cultures which had been incubated 18 to 24 hours. Granular growth was rarely encountered.

Filtrate.—Ultrafiltration was carried out in the usual manner, aided by suction. Berkefeld V, Chamberland, and Seitz filters were employed. Sterility of the filtrates was always determined by cultures.

Anticoagulant.—Potassium oxalate has been regularly employed in amounts of 0.02 gm. of oxalate to 10 cc. of blood. A 2 per cent solution is made in distilled water. 1 cc. of this solution is placed in small bottles which are heated in a dry air sterilizer until all water has evaporated. 10 cc. of blood, immediately after withdrawal, is mixed with the dried powder. 0.03 to 0.04 gm. of oxalate per 10 cc. of blood is used to obtain rabbit plasma.

Preparation of Fibrinogen.—The fraction of plasma proteins precipitated at 25 per cent saturation with ammonium sulfate has proved to be a suitable fibrinogen solution. To oxalated plasma, a saturated solution of ammonium sulfate is added drop by drop until the amount of ammonium sulfate equals one-third the volume of plasma. The mixture is stirred constantly during the addition of ammonium sulfate.

The flocculated protein is collected by centrifugation, and the supernatant liquid is discarded. The precipitate is washed once with 25 per cent saturated ammonium sulfate solution, centrifuged, and drained free of wash liquor. The protein is then dissolved in M/100 phosphate buffered physiological salt solution (pH 7.4). The amount of solvent which is added, makes the final protein solution equal the volume of plasma originally employed.

Preparation of Thrombin.[1]—The method consists of two stages.

First, precipitation of prothrombin from plasma. Oxalated plasma is diluted with a tenfold volume of cold, distilled water and maintained at ice bath temperature while carbon dioxide is passed through the mixture. After 10 minutes this operation is interrupted and the flocculent protein collected by centrifugation. The precipitate is dissolved in a volume of physiological salt solution slightly less than the volume of plasma originally employed. The hydrogen ion concentration of this solution is adjusted to pH 7.4 by means of a trace of solid sodium bicarbonate. This method separates prothrombin from the plasma but also precipitates some fibrinogen and other plasma proteins.

Second, conversion of prothrombin into thrombin. A 2.5 per cent solution of calcium chloride is added in a ratio of 1 cc. for each 10 cc. of prothrombin solution. After 5 to 10 minutes the solution coagulates to a thick gel. By careful manipulation the fibrin is rolled into a compact mass, and the expressed fluid is filtered through paper. This faintly opalescent solution usually possesses a high coagulating activity with either plasma or fibrinogen.

Fibrinogen and thrombin prepared according to the methods just described are sometimes labile and may deteriorate rapidly. Although some lots of each have maintained the original property for several days when kept in the ice box, it is best to use the materials on the day of preparation. In making tests for fibrinolysis, the physiological activity of both fibrinogen and thrombin was first established. Activity was deemed satisfactory when 0.5 cc. of undiluted fibrinogen formed a solid clot with 1 drop of thrombin solution.

Coagulants. 1. Thrombin.—0.1 cc. of thrombin solution was used to coagulate 0.2 cc. of plasma or fibrinogen. This ratio always insured complete clot formation in 1 to 3 minutes.

2. Calcium Chloride.—0.25 cc. of a 0.25 per cent solution of $CaCl_2$ in 0.85 per cent salt solution consistently clotted 0.2 cc. of oxalated plasma in 8 to 20 minutes. 0.3 to 0.35 cc. of 0.25 per cent $CaCl_2$ solution was added to rabbit plasma since a larger amount of oxalate was mixed with the whole blood. The solution of calcium chloride was sterilized by immersing in boiling water for 30 minutes.

Oxalated plasma which has stood for 3 days or more is rarely coagulable with $CaCl_2$. A distinct delay in clotting is usually noted in 2 day old plasma. The inability of calcium to coagulate old oxalated plasma is usually attributed to the rapid deterioration of prothrombin; the addition of thrombin to old plasma results in the formation of solid clot.

In the experiments reported in this article samples of plasma were always tested within 24 hours of the time of blood withdrawal.

Description of Test.—After numerous trials with varying amounts of the different constituents, the following standard procedure was adopted: 0.2 cc. of oxalated

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[1] The authors are indebted to Dr. Harry Eagle for the method by which thrombin solutions were prepared. The description, presented here, is given with his kind permission. A more detailed report will be published by Dr. Eagle.

plasma is diluted with 0.8 cc. of physiological salt solution. To this 1 to 5 dilution of plasma, 0.5 cc. of broth culture or sterile filtrate of test organism is added and well mixed. Coagulant is then added and well mixed; if $CaCl_2$ is used, 0.25 cc. of 0.25 per cent solution is added, if thrombin is employed, 0.1 cc. is added. The tubes are immediately placed in water bath at 37.5°C. The time at which solid coagulation occurs is noted. Solid coagulation is considered to be effected when the tube can be inverted without affecting the solid form of clot which adheres to the bottom and sides of the tube; usually no fluid, or only a small drop, escapes from the solid clot on inversion of the tube.

The tubes are allowed to remain in the water bath under continual observation. Complete dissolution of the clot is recorded as the time at which all evidence of fibrin has disappeared, and the contents of the tube are completely fluid. As dissolution begins, the first softening of the clot may be noted by gently shaking the tubes. This movement causes a wave-like motion to pass vertically down through the body of the clot. The coagulum then releases itself from the sides of the tube and usually forms a small mass which, a few minutes later, may be made to disappear by moderate agitation. All tests in which the plasma clot was resistant to dissolution were arbitrarily terminated after 24 hours incubation.

In the tables presented in this communication "complete dissolution" refers to the interval of time between formation of solid clot and complete liquefaction. The time necessary for clot formation is not given special consideration in this record.

By recapitulation, a test is as follows:

0.2 cc. plasma (or fibrinogen) + 0.8 cc. physiological salt solution + 0.5 cc. culture (or filtrate) + 0.25 cc. $CaCl_2$ (or 0.1 cc. thrombin).

Unless otherwise specified, all tests were done with standard quantities of the various constituents.

Fibrinolytic Tests with Human Strains of Hemolytic Streptococci

Twenty-eight strains of hemolytic streptococci of the *beta* type (Brown (1)) have been tested for the capacity to liquefy normal human plasma clot. The results are recorded in Table I.

From the table it can be seen that cultures of all the strains contain substances capable of causing fibrinolysis. Most of the strains effect complete dissolution in less than 20 minutes. With others the speed of clot-liquefaction varies but every strain of hemolytic streptococci derived from patients contains the active principle. Up to the present time no relationship has been noted between the disease source of organisms and fibrinolytic potency.

As noted in the table some of the strains were tested immediately after isolation while others have been in laboratory cultivation for

TABLE I

Strains of Hemolytic Streptococcus Isolated from Patients
Fibrinolytic Activity against Human Fibrin Clot

No.	Patient	Source of culture	Disease	Length of laboratory cultivation	Complete dissolution	
					Clot of	
					Plasma*	Fibrinogen†
1	Co.	Blood	Septicemia	2 yrs.	10 min.‡	2 min.‡
2	Le.	"	"	2 "	10 "	2 "
3	Ma.	"	"	2 "	20 "	
4	Bo.	"	"	1 wk.	10 "	2 "
5	Ot.	Throat	Acute tonsillitis	2 yrs.	15 "	
6	Hi.	"	" "	4 days	40 "	5 "
7	Bi.	"	" "	4 "	10 "	2 "
8	Hu.	"	" "	4 "	10 "	
9	Ja.	"	Scarlet fever	1 yr.	30 "	
10	Th.	"	" "	6 mos.	40 "	
11	Sh.	"	" "	6 "	10 "	
12	Mi.	"	" "	1 mo.	10 "	2 "
13	Ur.	"	" "	2 days	50 "	6 "
14	Si.	"	" "	2 "	10 "	2 "
15	Po.	"	" "	2 "	40 "	4 "
16	Pr.	"	" "	2 "	15 "	2 "
17	Zb.	"	" "	2 "	50 "	6 "
18	Shy.	"	" "	2 "	40 "	6 "
19	Ch.	Ear	Otitis media	2 "	20 "	3 "
20	La.	Throat	Erysipelas	2 wks.	10 "	
21	Br.	"	"	1 mo.	10 "	
22	Ro.	Skin	"	6 mos.	25 "	
23	Pr.	Pleural fluid	Empyema	1 wk.	10 "	2 "
24	Sm.	Tonsil	Acute nephritis	7 yrs.	10 "	
25	Ta.	"	" "	7 "	15 "	
26	Mc.	Throat	" "	7 "	10 "	
27	Ba.	Skin pustule	" "	2 mos.	10 "	2 "
28	Lab.	Skin	Cellulitis	3 "	20 "	

All tubes incubated in water bath at 37.5°C.

* Plasma clotted with $CaCl_2$

† Fibrinogen clotted with human thrombin.

‡ Time indicated is interval between formation of solid clot and complete liquefaction. Repeated tests have been made with many of the strains. The average rate of dissolution is noted in the table.

several years. Strain Co., continually highly active for 12 months, has been used in most of the tests. This strain was originally isolated by blood culture from a fatal case of septicemia and has been carried in the laboratory for 2 years.

Although fibrinolytic activity is maximum in freshly transplanted cultures which have been incubated 18 to 24 hours, the property may be retained for several days. Deterioration of the active principle supplied by the organisms, however, is noticeable within 1 to 2 weeks, even at ice box temperature. Consequently, in an attempt to obtain a maximum uniform effect, broth cultures were always used the day following inoculation. In spite of the fact that this procedure necessitated the use of fresh subcultures frequently, the yield of active material maintained a constant high level.

In addition to tests performed with the clot of whole plasma, fibrinogen has been chemically removed from plasma and used as a source of fibrin. Coagulation, in these instances, has been induced by the addition of thrombin solutions, also derived from whole blood. The fibrin formed by the combination of chemically isolated fibrinogen and thrombin solution is called fibrinogen clot in this article, to distinguish it from the clot of whole plasma coagulated by calcium which is referred to as plasma clot. The fibrinogen clot, consisting of fibrinogen plus active culture plus thrombin, has been observed for fibrinolysis in exactly the same manner as that employed with plasma clot.

The results of tests with 15 strains of hemolytic streptococcus against fibrinogen clot are given in Table I and demonstrate that with this material, fibrinolytic activity proceeds with greater rapidity than it does against the fibrin of whole plasma. The culture of Strain Co., for example, which liquefied plasma clot in 10 minutes, dissolved a relatively comparable amount of fibrinogen clot in 2 minutes. The exact amount of fibrin in the plasma clot and the fibrinogen clot was not determined and the comparable experiments recorded in the table indicate only the qualitative character of the phenomenon rather than accurate quantitative titrations, which will be subsequently published. That whole plasma contains a property which may delay or even inhibit the fibrinolytic activity of the organisms will be referred to later in this article.

Fibrinolytic Tests with Animal Strains of Hemolytic Streptococci

In addition to the 28 strains of hemolytic streptococci derived from patients, 18 strains from animal sources have been similarly tested for the capacity to liquefy coagulated plasma and fibrinogen. These strains are hemolytic streptococci of the *beta* type and equally as active in producing hemolysis as the human strains. The results of tests with broth culture of the 18 animal strains against human fibrin clot are recorded in Table II.

TABLE II

Animal Strains of Hemolytic Streptococcus
Fibrinolytic Activity against Human Fibrin Clot

No.	Strain	Animal source	Complete dissolution	
			Clot of	
			Plasma*	Fibrinogen†
1	K 226	Rabbit	Neg. 24 hrs.‡	Neg. 24 hrs.‡
2	K 158A	"	" 24 "	" 24 "
3	K 158B	"	" 24 "	" 24 "
4	K 158C	"	*30 min.*	*8 min.*
5	K 158D	"	Neg. 24 hrs.	Neg. 24 hrs.
6	K 158E	"	" 24 "	" 24 "
7	K 158F	"	" 24 "	" 24 "
8	V 10	Cow	*10 min.*	*5 min.*
9	K 56	Guinea pig	Neg. 24 hrs.	Neg. 24 hrs.
10	K 61	" "	*30 min.*	*8 min.*
11	K 64	" "	Neg. 24 hrs.	Neg. 24 hrs.
12	K 104	" "	" 24 "	" 24 "
13	P 454	" "	" 24 "	" 24 "
14	J 20	" "	" 24 "	" 24 "
15	Br 1	Rabbit	" 24 "	" 24 "
16	Br 2	Guinea pig	" 24 "	" 24 "
17	H 1	Horse	" 24 "	" 24 "
18	R 1	Rabbit	" 24 "	" 24 "

All tubes incubated in water bath at 37.5°C.

* Plasma clotted with $CaCl_2$.

† Fibrinogen clotted with human thrombin.

‡ Time indicated is interval between formation of solid clot and complete liquefaction. All experiments terminated after 24 hours incubation.

Strains 1 to 14 were kindly supplied by Dr. R. C. Lancefield, Hospital of The Rockefeller Institute, New York.

Strains 15 and 16 were kindly supplied by Dr. J. Howard Brown, Department of Pathology and Bacteriology, Johns Hopkins Medical School, Baltimore.

From the table it can be seen that 15 of these strains are totally incapable of inducing clot liquefaction even though the method of experimental procedure was identical with that used in testing the human strains.

Concerning the 3 strains of animal source which liquefied human clot, it is interesting to record that their cultivation on blood agar yielded, with each of the strains, two distinct types of colony; one type was small with a wide zone of hemolysis; the other type was larger, tended to be mucoid, and had a relatively smaller zone of hemolysis. When these two types of colonies were isolated and grown in broth, the small colony yielded a culture which was inert against clot, whereas broth transplant of the larger type of colony was highly active in dissolving coagulated plasma and fibrinogen. The significance of this behavior of the culture is not yet understood. The 15 strains of animal origin which were inactive produced colonies similar to the smaller, strongly hemolytic type.

The result of the observations with animal strains is interesting in that it demonstrates that the capacity to liquefy human clot is absent or suppressed in some strains of *Streptococcus hemolyticus* even though the organisms conform in other cultural respects to active strains; fibrinolysis does not necessarily parallel hemolysis.

Fibrinolytic Tests with Other Human Bacterial Species

In order to determine whether or not the fibrinolytic substances are limited to hemolytic streptococci, comparable tests have been performed with other pathogenic bacteria derived from patients.

All of the tests have been completely negative. The list of other organisms consists of:

6 strains of *Streptococcus viridans.*
2 strains of non-hemolytic streptococci.
6 strains of streptococci with weak hemolyzing property, some of which have been identified as *alpha* prime (Brown (1)) variety.
12 strains of pneumococci of different types (two of Type I strain, one of Type II, two of Type III, one each of Types VI, VIII, and XIV, three untyped Group IV strains, and one R strain of pneumococcus originally derived from Type II).
2 strains of typhoid bacillus.

2 strains of colon bacillus.
1 strain each of paratyphoid bacillus A and B.
1 strain each of Shiga and Flexner dysentery bacillus.
2 strains of Freidländer bacillus, Types A and B (Julianelle (2)).
2 strains of hemolytic influenza bacillus.

Altogether 38 strains of organisms other than hemolytic streptococci have been examined and in no instance have any of these cultures affected either clotted plasma or the more susceptible fibrinogen clot during the 24 hour test period.

It will be noted that strains of *Staphylococcus aureus* and *Staphylococcus albus* are not included in the list. Interesting properties by which cultures of staphylococcus affect plasma have been reported by Much (3), Gratia (4), Gross (5), and others. They have found that these organisms are capable of coagulating plasma spontaneously and some of the articles have reported that liquefaction may also occur over a period of several hours. These properties of staphylococci are being investigated separately in this laboratory. Up to the present time, fibrinolytic activity has been found in only occasional strains and, when present, causes dissolution irregularly and requires 18 to 24 hours or longer to act.

With the possible exception, then, of staphylococci, hemolytic streptococci appear to be unique among the species of bacteria usually pathogenic for human beings in the capacity to transform rapidly, solid clotted fibrin into a thin fluid state.

Fibrinolytic Tests with Sterile Filtrates of Hemolytic Streptococci

The observations so far described have been made by adding to plasma, whole broth cultures containing both living organisms and the bacterial products present in the medium. Additional investigations have been carried out with sterile, cell-free filtrates, with washed organisms, and with cultures grown on solid media.

The sterile filtrates of 6 strains of hemolytic streptococcus, grown in broth, have been tested for the presence of fibrinolytic substances by the use of human plasma clot and also human fibrinogen clot. The results are presented in Table III. Filtration has been made through Berkefeld V, Chamberland, and Seitz filters. The records contained in the table were obtained with material passed through Berkefeld V candles.

From the table it may be seen that, in most instances, 0.5 cc. of cell-free filtrate liquefies normal human fibrin clot as rapidly as does 0.5 cc. of whole broth culture; with less active strains, the filtrate acts at a slightly slower rate than does whole culture. The presence of the fibrinolytic substances in the filtrate evidences the fact that the active principle is elaborated by growing organisms and is liberated freely into the surrounding medium.

TABLE III

Fibrinolytic Activity of Sterile Filtrates of Active Strains of Hemolytic Streptococcus against Human Fibrin Clot

Strains	Amount of filtrate	Amount of whole broth culture	Complete dissolution Clot of Plasma*	 Fibrinogen†
	cc.	*cc.*		
Co.	0.5	—	10 min.‡	2 min.‡
	—	0.5	10 "	2 "
Le.	0.5	—	10 "	2 "
	—	0.5	10 "	2 "
Br.	0.5	—	12 "	2 "
	—	0.5	10 "	2 "
Ba.	0.5	—	15 "	4 "
	—	0.5	10 "	2 "
Ma.	0.5	—	30 "	6 "
	—	0.5	20 "	4 "
Hi.	0.5	—	1 hr. 10 min.	10 "
	—	0.5	40 min.	8 "

All tubes incubated in water bath at 37.5°C.

* Plasma clotted with $CaCl_2$.

† Fibrinogen clotted with human thrombin.

‡ Time indicated is interval between formation of solid clot and complete liquefaction. Repeated tests have been made with many of the strains. The average rate of dissolution is noted in the table.

Titration of Fibrinolytic Activity of Sterile Filtrates

When whole broth cultures were employed in attempts to titrate quantitatively the amount of fibrinolytic substance present, the presence of living organisms detracted from the accuracy of the results. In such attempts the additional multiplication of bacterial cells which might take place at 37.5°C. was uncontrolled. By the use of sterile filtrates, however, the degree of activity has been more satisfactorily estimated.

Titrations of filtrates have been done both by testing varying dilutions of filtrate against a constant quantity of plasma and fibrinogen, and by testing a constant quantity of filtrate against varying amounts of plasma and fibrinogen. The results are presented in Table IV.

The observations given in the table demonstrate that, against the clot contained in 0.2 cc. plasma, 0.01 cc. of filtrate effects fibrinolysis almost as rapidly as does 0.5 cc. of filtrate. 0.005 cc. is slowly active, whereas 0.001 cc. is ineffectual. When progressively decreasing quantities of filtrate were tested against fibrinogen clot, even so small an amount as 0.0005 cc. of filtrate induced lysis in an hour and 5 minutes. Smaller quantities of filtrate have not been tested. The greater susceptibility of fibrinogen-clot is also brought out in the lower half of Table V, where the increasing amounts of fibrin substrate are exposed to a constant quantity of active filtrate. In these experiments, 0.5 cc. of filtrate required 3 hours and a half to liquefy 2 cc. of plasma clot; whereas 0.5 cc. of filtrate dissolved 10 cc. of fibrinogen clot in 30 minutes.

The titration of many lots of active filtrate has resulted in some variation in quantitative activity. The results given in Table IV were obtained with a highly active product; in a few instances even more potent material has been obtained.

When organisms are removed from broth by centrifugation, washed, and resuspended in physiological salt solution, their capacity to dissolve fibrin clot is considerably delayed. In such experiments liquefaction required 6 to 10 hours. This was interpreted as being chiefly due to the fact that most of the preformed fibrinolytic property was discarded, and that the slow rate of liquefaction depended upon the speed with which additional active material could be formed by the organisms and excreted into the clot. A similar delayed rate of dissolution occurred when streptococci grown on blood agar were washed off and tested for fibrinolytic activity.

TABLE IV

Titration of Fibrinolytic Activity of Sterile Filtrate against Human Fibrin Clot

Amount of filtrate	Amount of plasma*	Time of complete dissolution	Amount of filtrate	Amount of fibrinogen†	Time of complete dissolution
Decreasing amounts of filtrate against constant quantity of plasma clot and fibrinogen clot					
cc.	*cc.*		*cc.*	*cc.*	
0.5	0.2	8 min.‡	0.5	0.2	2 min.‡
0.1	0.2	10 "	0.1	0.2	2 "
0.05	0.2	10 "	0.05	0.2	4 "
0.01	0.2	30 "	0.01	0.2	7 "
0.005	0.2	6 hrs.	0.005	0.2	10 "
0.001	0.2	Neg. 24 hrs.	0.001	0.2	40 "
0.0005	0.2	—	0.0005	0.2	11 hr. 5 min.
Constant amount of filtrate against increasing quantities of plasma clot and fibrinogen clot					
0.5	0.2	7 min.	0.5	0.2	2 min.
0.5	0.5	15 "	0.5	0.5	2 "
0.5	1.0	30 "	0.5	1.0	3 "
0.5	2.0	3 hrs. 30 min.	0.5	2.0	4 "
0.5	5.0	20 hrs.	0.5	5.0	5 "
0.5	10.0	—	0.5	10.0	30 "

All tubes incubated in water bath at 37.5°C.

* Plasma clotted with $CaCl_2$.

† Fibrinogen clotted with human thrombin.

‡ Time indicated is interval between formation of solid clot and complete liquefaction.

In each instance the plasma and fibrinogen was diluted 5 times. The amount of coagulant necessary was calculated on the basis of a standard test (see pages 487 and 488).

That the active substances contained in broth cultures may be separated from the living bacterial cells has facilitated chemical studies on the nature of the fibrinolytic property elaborated by hemolytic streptococci. Even though the interaction between cultural material and fibrin is comparable to enzyme activity, the fibrinolytic principle has special characteristics different from the metabolic proteolytic enzymes of hemolytic streptococci as described by Tongs (6), West and Stevens (7), and Frobisher (8). The investigation has, therefore, been extended to include a study of the nature and properties of the active substance. A detailed report will follow this preliminary record.

Resistance to Fibrinolysis by Plasma of Patients Recovered from Hemolytic Streptococcus Infections

In addition to observations on the occurrence and properties of the fibrinolytic substance, the blood of patients and of normal individuals has been investigated for the purpose of determining whether or not infection with hemolytic streptococci is followed by changes in plasma which are demonstrable by tests for susceptibility to fibrinolysis. Tests have, therefore, been made on samples of plasma taken at repeated intervals before and after recovery from infection. The results demonstrate that the plasma clot from the blood of patients recovered from hemolytic streptococcus diseases is often completely resistant to dissolution by active culture. An analysis of many tests on samples of blood taken during the course of disease is now in preparation and will form a separate communication. At the present time it is interesting to record an instance of resistance and also to note the fact that the serum of an individual, whose plasma is resistant, may render normal plasma clot insusceptible.

An example of the resistant effect is given in the following protocol, which consists of tests done with the plasma and serum of a patient convalescent from erysipelas.

1. Patient's plasma + culture + $CaCl_2$: Negative 24 hours.
2. Normal plasma + culture + $CaCl_2$: Complete dissolution in 10 minutes.
3. Normal plasma + culture + 0.1 cc. patient's serum + $CaCl_2$: No dissolution in 24 hours.
4. Normal plasma + culture + 0.1 cc. normal serum + $CaCl_2$: Complete dissolution in 10 minutes.

Standard quantities of constituents were employed in the experiment.

Resistance to the fibrinolytic activity of hemolytic streptococci exemplified in the protocol just given has been observed with the plasma of other convalescent patients. The absence of fibrinolysis may also be strikingly demonstrated by the use of normal animal plasma.

Resistance to Fibrinolysis by Plasma of Rabbits

Table V contains the results of fibrinolytic tests done on rabbit plasma clot and rabbit fibrinogen clot with 6 human strains and 3 animal strains of hemolytic streptococci highly active against human clot, and with 3 rabbit strains inactive against human fibrin.

TABLE V

Fibrinolytic Activity of Human and Animal Strains of Hemolytic Streptococcus against Rabbit Fibrin Clot

No.	Strain	Source	Activity against human fibrin clot	Rabbit fibrin clot Complete dissolution Clot of Plasma*	 Fibrinogen†
1	Co.	Human	++++	Neg. 24 hrs.‡	Neg. 24 hrs.‡ or 10-20 hrs. Complete
2	Le.	"	++++	" 24 "	" "
3	Br.	"	++++	" 24 "	" "
4	Ba.	"	++++	" 24 "	Neg. 24 hrs.
5	Hu.	"	++++	" 24 "	" 24 "
6	Si.	"	++++	" 24 "	" 24 "
7	K 158A	Rabbit	Neg.	" 24 "	" 24 "
8	K 158B	"	"	" 24 "	" 24 "
9	K 158C	"	+++	" 24 "	" 24 "
10	K 226	"	Neg.	" 24 "	" 24 "
11	K 61	"	++++	" 24 "	" 24 "
12	V 10	Cow	++++	" 24 "	" 24 "

All tubes incubated in water bath at 37.5°C.

* Clotted with $CaCl_2$.

† Clotted with rabbit thrombin.

‡ Experiments arbitrarily terminated at end of 24 hour incubation.

++++ indicates maximum activity (see Table I).

The complete insusceptibility of rabbit plasma clot to the fibrinolytic property of the organisms contrasts strikingly to the rapid liquefaction of human clot. Plasma from fifteen adult rabbits and three young rabbits, 1 month old, has been tested in order to determine individual variation. The results have been uniformly negative.

The coagulation of separated rabbit fibrinogen, in the presence of active culture, by rabbit thrombin results in the formation of a fibrinogen clot which, in most instances, has been as resistant to dissolution as the plasma clot. Exceptions to this experience have been encountered, however, which are indicated in Table V by the record of dissolution of fibrinogen clot in 10 to 20 hours. That rabbit fibrinogen clot may occasionally be slowly liquefied, whereas whole rabbit plasma clot is not, indicates that the factor responsible for resistance is greater in whole plasma than in the constituents which have been chemically isolated. The behavior of the rabbit fibrinogen-thrombin mixtures, although not identical with the results obtained with human material, tends to conform with the results presented in Table I. An understanding of the phenomenon must await an analysis of the factors which influence the fibrinolytic process.

Relation of Thrombin to Resistance

Additional evidence as to the complexity of the mechanism which determines the susceptibility or resistance of clot to dissolution is brought out in experiments in which coagulation is induced by different agents. In the observations so far presented human plasma has been clotted by the addition of $CaCl_2$. By this method, calcium promotes the transformation of the prothrombin of the plasma into thrombin, which in turn changes the fibrinogen into fibrin. The whole process of coagulation is effected by homologous native agents. Similarly rabbit plasma clotted with calcium behaves in an identical manner. Where fibrinogen has been used for fibrin clot, coagulation has been effected by homologous thrombin.

Interesting differences in the susceptibility of clot to the fibrinolytic substance of streptococci are brought out by interchanging human and animal materials which go to form the fibrin clot. The results are contained in the protocol given below. In all of the tests listed, the coagulation was complete and, from the gross appearance of the clot, the occurrence of fibrinolysis could not be anticipated. The experiments were controlled with tests, in which physiological salt plasma of other convalescent patients. The absence of fibrinolysis may also be strikingly demonstrated by the use of normal animal plasma.

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2	Le.	"	++++	" 24 "	" "
3	Br.	"	++++	" 24 "	" "
4	Ba.	"	++++	" 24 "	Neg. 24 hrs.
5	Hu.	"	++++	" 24 "	" 24 "
6	Si.	"	++++	" 24 "	" 24 "
7	K 158A	Rabbit	Neg.	" 24 "	" 24 "
8	K 158B	"	"	" 24 "	" 24 "
9	K 158C	"	+++	" 24 "	" 24 "
10	K 226	"	Neg.	" 24 "	" 24 "
11	K 61	"	++++	" 24 "	" 24 "
12	V 10	Cow	++++	" 24 "	" 24 "

All tubes incubated in water bath at 37.5°C.

* Clotted with $CaCl_2$.

† Clotted with rabbit thrombin.

‡ Experiments arbitrarily terminated at end of 24 hour incubation.

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Interesting differences in the susceptibility of clot to the fibrinolytic substance of streptococci are brought out by interchanging human and animal materials which go to form the fibrin clot. The results are contained in the protocol given below. In all of the tests listed, the coagulation was complete and, from the gross appearance of the clot, the occurrence of fibrinolysis could not be anticipated. The experiments were controlled with tests, in which physiological salt solution or sterile uninoculated broth was substituted for active filtrate. The clot, formed under these conditions, remained intact.

				Clot formation	Time of Clot dissolution
1.	Human plasma	+ filtrate	+ CaCl$_2$	10 min.	10 min.
2.	" "	+ "	+ human thrombin	1 "	6 "
3.	" "	+ "	+ rabbit "	1 "	10 "
4.	Rabbit plasma	+ filtrate	+ CaCl$_2$	10 "	Neg. 24 hrs.
5.	" "	+ "	+ human thrombin	1 "	45 min.
6.	" "	+ "	+ rabbit "	1 "	Neg. 24 hrs.
7.	Human fibrinogen	+ filtrate	+ human thrombin	1 "	2 min.
8.	" "	+ "	+ rabbit "	1 "	4 "
9.	Rabbit fibrinogen	+ filtrate	+ human thrombin	1 "	4 "
10.	" "	+ "	+ rabbit "	1 "	Neg. 24 hrs. or complete dissolution in 10–20 hrs.

From this list it can be seen that when the constituents of the clot are composed of human material, liquefaction occurs promptly. Fibrinolysis is rapidly induced by filtrate whether the fibrinogen and thrombin are resident in whole plasma or are first chemically separated and then recombined. On the contrary, when the coagulum is made up entirely of rabbit substances, the clot remains intact. An exception to this is found in the few instances, already referred to, where rabbit fibrinogen plus rabbit thrombin has produced a clot which is finally dissolved after 10 to 20 hours. The use of human thrombin in the coagulation of rabbit plasma or fibrinogen, however, results in the formation of a clot which active filtrate is able to liquefy with great rapidity. Repeated observations have uniformly demonstrated that the addition of a clot constituent from a susceptible blood to a esistant blood results in a coagulum which active cultural material an successfully liquefy.

At the present time the investigation has not proceeded far enough o permit discussion of the mechanism of susceptibility and resistance hich is manifest under the experimental conditions just described. he fibrinogen and thrombin preparations are not purified and unoubtedly contain other blood constituents, the importance of which as yet undetermined. The observations have, however, been repeatedly made and the facts, which have been established, serve as a basis for additional investigations.

SUMMARY

Broth cultures of hemolytic streptococci derived from patients are capable of rapidly liquefying normal human fibrin clot.

The active fibrinolytic principle is also contained in sterile, cell-free filtrates of broth cultures. The degree of activity of filtrates parallels the activity of whole broth cultures sufficiently closely to indicate that large amounts of the fibrinolytic substance are freely excreted into the surrounding medium and pass readily through Berkefeld V, Seitz, and Chamberland filters.

The occurrence of fibrinolysis is most strikingly observed when plasma or fibrinogen is mixed with active cultural material before clot formation is effected. Under the standard experimental conditions described, complete dissolution of human plasma clot (whole oxalated plasma + $CaCl_2$) occurs in about 10 minutes; complete dissolution of human fibrinogen clot (chemically isolated fibrinogen + thrombin) takes place in about 2 minutes. Titration of filtrate activity is recorded in Table IV.

Twenty-eight strains of *Streptococcus hemolyticus*, isolated from patients suffering from various manifestations of streptococcus infection, have been tested for the capacity to liquefy fibrin clot. Broth cultures of all of the strains induced fibrinolysis.

Of 18 strains of *Streptococcus hemolyticus* of animal origin, only three were capable of causing dissolution of clot.

Completely negative results were obtained with 38 strains of other bacterial species. The list is presented on pages 492 and 493.

The plasma of many patients recovered from acute hemolytic streptococcus infections, when clotted in the presence of active cultures, is highly resistant to fibrinolysis. Furthermore, serum, derived from patients whose plasma clot is resistant, often confers on normal plasma clot an antifibrinolytic property. One example of the resistance possessed by the blood of convalescent patients is presented in this report. A second paper, now in preparation, will give in detail a large number of observations on the relation of infection to the development of resistance to the fibrinolytic activity of hemolytic streptococci.

In contrast to the susceptibility of normal human fibrin clot to liquefaction by active culture, normal rabbit fibrin clot is totally resistant to dissolution when tested under comparable conditions. The insusceptibility of rabbit fibrin clot is manifest provided the coagulum is composed of rabbit constituents. When human thrombin is used to clot rabbit plasma or fibrinogen in the presence of active cultures, fibrinolysis is not prohibited. The rôle of thrombin in determining the resistance or susceptibility of rabbit fibrin to dissolution offers a suggestive approach to problems relating to the underlying mechanism.

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THE RELATION OF VITAMIN C DEFICIENCY TO INTESTINAL TUBERCULOSIS IN THE GUINEA PIG

By MACK McCONKEY, M.D., and DAVID T. SMITH, M.D.

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Plates 26 to 28

(Received for publication, June 27, 1933)

Intestinal tuberculosis is one of the most frequent and serious complications of phthisis. Up to the present time attempts at prevention and treatment have been empirical and unsatisfactory, because the factors involving the production of the intestinal ulcers have not been well understood. The swallowing of sputum laden with bacilli cannot be the sole cause of the condition since many patients swallow such sputum in large quantities for years without developing intestinal lesions, and the problem resolves itself with the question of why the intestines of some patients are susceptible and others resistant to the bacilli passing through them.

Numerous attempts have been made in the past by many workers to produce intestinal tuberculosis in the guinea pig by feeding either tubercle bacilli or tuberculous material. As a rule, the animals develop enlarged cervical or mesenteric lymph glands and die of generalized tuberculosis without developing ulcers in the intestinal tract, but success has been claimed by a few workers. From the reports of the following investigations it is difficult to determine whether or not the diets of their experimental animals were adequate in vitamins.

Villemin (8) in 1868 fed 40 gm. of tuberculous sputum to guinea pigs and killed the animals 3 months later. Lesions were found in the small bowel and cecum. Klebs (4) in 1870 fed guinea pigs tuberculous matter and found tuberculous ulceration in the intestines in two animals. Redman (7) in 1922 reported that in every instance in which he fed virulent tubercle bacilli to guinea pigs, the mesenteric lymph nodes became involved in 34 to 41 days. There were tuberculous nodules in the cecum and colon at 81 and 86 days. They were not open ulcers and no microscopic examinations were reported. Medlar and Sasano (6) in 1924 inocumothers showed no evidence of scurvy but the new-born pigs showed marked signs of this disease. The remaining three animals were killed in the 5th month at the end of the experiment and found to be normal.

Group D. Six normal adult pigs received a basic diet plus 0.3 cc. of cod liver oil and 10 cc. of tomato juice daily. These animals did well for 2 months. Two pigs died of spontaneous pulmonary disease between the 2nd and the 3rd month, and the remaining four were killed in the 5th month at the end of the experiment and found to be normal.

2. Twenty-six adult guinea pigs (Groups E, F, G, and H) were used to determine the effect of various diets on tuberculous animals. Each pig was sensitized by a subcutaneous injection of non-virulent human tubercle bacilli (No. R1 Saranac Lake Laboratory) and subsequently fed 0.5 cc. of tuberculous sputum with a tuberculin syringe daily.

Group E. Ten of the above pigs received a basic diet and 0.5 cc. of tuberculous sputum daily. These animals died between the 2nd and 5th months. Three animals which died early were found to have a normal intestinal tract. Five animals presented one or more open tuberculous ulcers in the intestines and two animals showed caseous tubercles in the lymph follicles of the ileum which had not ulcerated. The ulcerative lesions were most common in the ileum, with cecum next, and jejunum and colon least frequently involved.

Group F. Six sensitized adult pigs received 0.5 cc. of tuberculous sputum daily, and were fed in addition to the basic diet 0.3 cc. of cod liver oil daily. These animals died between 6 weeks and 4 months. One animal had a normal intestinal tract, and one had non-ulcerated caseous tubercles in the ileum, while the remaining four presented from one to twenty open tuberculous ulcers in the ileum, cecum, jejunum, and colon.

Group G. Five sensitized adult pigs received 0.5 cc. of tuberculous sputum daily, and were fed in addition to the basic diet, 10 cc. of tomato juice daily. These animals all did well and were killed in the 5th month at the end of the experiment. One animal had a caseous tubercle in the ileum which had not ulcerated. The other four animals presented normal intestinal tracts, although there was extensive tuberculosis in the liver, spleen, and lungs.

Group H. Five sensitized adult pigs receiving 0.5 cc. of tuberculous sputum daily were fed in addition to the basic diet 0.3 cc. of cod liver oil and 10 cc. of tomato juice daily. These animals all did well except one which died of unknown causes in the 10th week. The remaining four were killed in the 5th month at the end of the experiment. The intestinal tracts of all five animals were normal although considerable tuberculosis was present in the liver, spleen, and lungs.

Discussion of Experiment I

Normal adult guinea pigs kept on the vitamin poor diet described died in from 6 to 16 weeks; some showed definite evidence of scurvy, while others presented a variety of lesions ascribable to the deficient liet. Cod liver oil did not prevent the development of these lesions, ut the addition of 10 cc. of tomato juice daily to the basic diet proected the animals completely from all signs of deficiency disease.

Sensitized guinea pigs maintained on the basic diet and fed 0.5 cc. of tuberculous sputum daily developed ulcerative tuberculous lesions in the intestines in five out of ten cases. When cod liver oil was added to the basic diet, four out of six animals developed ulcerative intestinal tuberculosis. When, however, the basic diet was supplemented with 10 cc. of tomato juice daily, the animals remained in good health and only one out of ten showed any trace of intestinal tuberculosis.

To recapitulate—of the sixteen sensitized guinea pigs on diets deficient in vitamin C or in vitamins C, A, and D, which received 0.5 cc. of tuberculous sputum daily, nine presented one or more open tuberculous ulcers of the intestines and three presented caseous non-ulcerative tubercles while only four animals presented normal intestines at necropsy. In contrast, nine out of ten guinea pigs presented normal intestinal tracts when the basic diet was supplemented either with tomato juice or cod liver oil and tomato juice. The remaining animal showed a few non-ulcerative tubercles in the ileum.

Experiment II. Mar. 15, 1929.—Forty-six normal adult pigs were used in this experiment (Groups I, J, K, L, and M). With the exception of ten animals in Group M, all the pigs received three doses of approximately 1 mg. of virulent human tubercle bacilli (No. H 37, Saranac Lake Laboratory) by mouth between Mar. 15 and 20. They were given our regular stock diet until Apr. 15 when a number of the pigs presented palpable glands in the neck. The animals were then divided into five groups.

Group I. Sixteen of these animals were given the basic diet described in Experiment I, and 0.5 cc. of tuberculous sputum daily. Eleven of the sixteen pigs died between the 6th week and the 3rd month. The remaining five were killed at the end of the 4th month. Necropsy showed that thirteen pigs had from one to ten tuberculous ulcers in the intestines, two presented caseous non-ulcerative tubercles in the ileum, and only one intestinal tract was normal. In addition, three of the animals had duodenal ulcers and one a pyloric ulcer, all of which were shown to be non-tuberculous on microscopic examination.

Group J. Five adult guinea pigs received 0.3 cc. of cod liver oil and 0.5 cc. of tuberculous sputum in addition to the basic deficient diet. Three of the five pigs died between the 2nd and 3rd months, the remaining two were killed at the end of the 4th month. One animal had numerous caseous non-ulcerative tubercles in the ileum and cecum, and the other four had from one to fifteen tuberculous ulcers in the intestines. One animal had a small duodenal ulcer that was not tuberculous.

that in the early scorbutic lesions submucosal hemorrhage was the outspoken feature; the mucosa overlying the area of hemorrhage becomes necrotic, sloughs, and forms an ulcer (Figs. 9–11). The initial tuberculous lesion also occurs in the submucosa (Figs. 2, 12) and apparently goes on to ulceration (Fig. 3) when there is not sufficient vitamin C in the diet. Apparently, an adequate supply of vitamin C in the diet protects the guinea pig against ulcerative intestinal tuberculosis, even in the presence of submucous tubercle.

The guinea pig and man appear to be similar in their vitamin C requirements. Patients with intestinal tuberculosis improve when tomato juice is added to their diet, while our tuberculous guinea pigs develop intestinal lesions unless protected by an adequate supply of vitamin C in their food.

Our experiments however, do not explain the clinical value of cod liver oil in intestinal tuberculosis. Man is quite susceptible to vitamin A and D deficiencies, while the guinea pig is not. It is thus possible that a deficiency of vitamins A and D (the vitamins of cod liver oil) plays a part in the development of intestinal tuberculosis in man and not in the guinea pig. Before this part of the problem can be settled it will be necessary to repeat our experiments utilizing an animal such as the monkey which is susceptible to the same vitamin deficiencies as man and also to tuberculosis.

CONCLUSION

1. Seventy-two adult guinea pigs were fed tuberculous sputum daily for periods ranging from 6 weeks to 4 months.
2. Thirty-seven of these were maintained on a diet partially deficient in vitamin C; twenty-six developed ulcerative intestinal tuberculosis.
3. In the remaining thirty-five animals whose diet was supplemented by an adequate amount of vitamin C only two developed tuberculous ulcers in the intestines.
4. From these studies we conclude that the ingestion of tubercle bacilli by the guinea pig is not the sole factor in the production of intestinal tuberculosis.
5. In our opinion, an adequate supply of vitamin C usually protects the guinea pig against ulcerative intestinal tuberculosis.

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EXPLANATION OF PLATES

Plate 26

Fig. 1. Photograph of tuberculous ulcers from the small intestine of a guinea pig. ×2.

Fig. 2. Low power photomicrograph of a submucosal tubercle in the intestine of a guinea pig. The mucosa is still intact. ×35.

Fig. 3. Low power photomicrograph of a chronic tuberculous ulcer in the intestines of a guinea pig. ×35.

Fig. 4. Low power photomicrograph of an early tuberculous involvement of a solitary follicle in a guinea pig. See Fig. 5. ×20.

Plate 27

Fig. 5. Camera lucida drawing of a section from early tuberculous ulceration of a solitary follicle in the intestines of a guinea pig. The tubercle bacilli are present only in monocytes. Some of the monocytes are degenerating. A low power photomicrograph of the section from which this drawing was made is shown in Fig. 4. Approximately ×800.

Fig. 6. Camera lucida drawing of a section from an early tuberculous ulcer in the intestines of a guinea pig. Tubercle bacilli are seen in monocytes and in giant cells. Note the dilatation of the capillaries and the proliferation of fibroblasts. Approximately ×800.

Fig. 7. Camera lucida drawing from a section of an extensive tuberculous ulcer in the intestines of a guinea pig. The superficial portion of the lesion is entirely necrotic. Beneath this layer one sees polymorphonuclear leucocytes in various stages of degeneration. Fibroblasts, lymphocytes, monocytes, and giant cells are seen in the depth of the lesion. Approximately ×800.

Fig. 8. Camera lucida drawing of a giant cell in a submucosal tubercle in the intestines of a guinea pig. Low power photomicrograph of the lesion from which this drawing was made is shown in Fig. 2. Approximately ×800.

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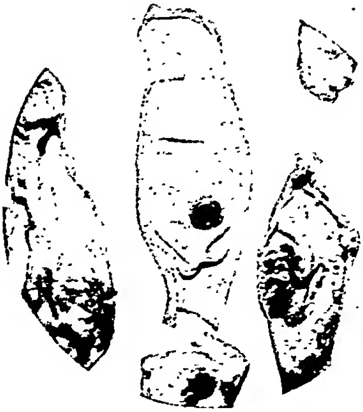
PLATE 28

FIG. 9. Low power photomicrograph of acute scorbutic ulcer in the ileum of a guinea pig. ×15.

FIG. 10. Low power photomicrograph of acute scorbutic ulcer in the ileum showing hemorrhage in mucosa and submucosa. ×90.

FIG. 11. High power photomicrograph of section shown in Fig. 10. Note marked degeneration of mucosa and absence of cellular reaction. ×390.

FIG. 12. High power photomicrograph of the mucosa overlying the caseous tuberculous lesion seen in Fig. 2. Note the accumulation of well preserved lymphocytes and monocytes in contrast to the ischemic necrosis found in the scorbutic lesion shown in Fig. 11. ×430.



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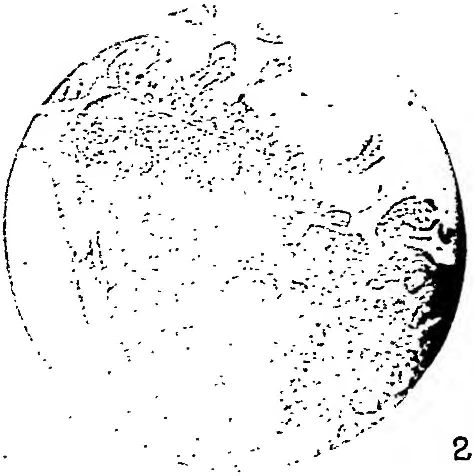

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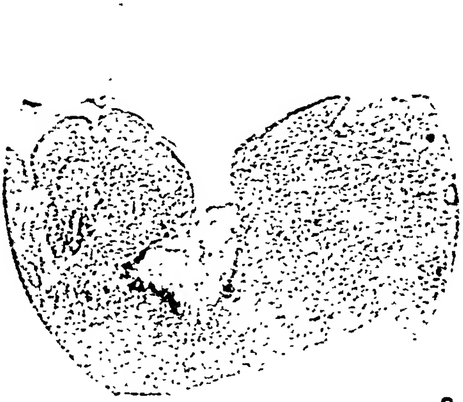

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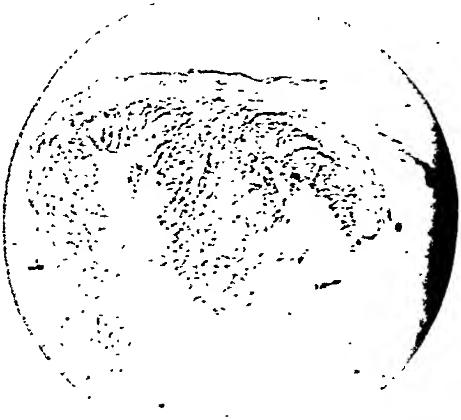

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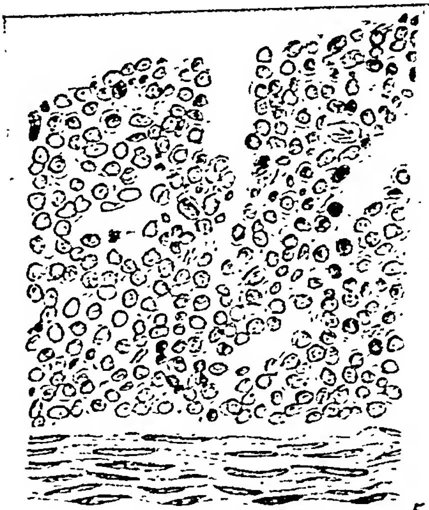
(McConkey and Smith: Vitamin C lack and intestinal tuberculosis)



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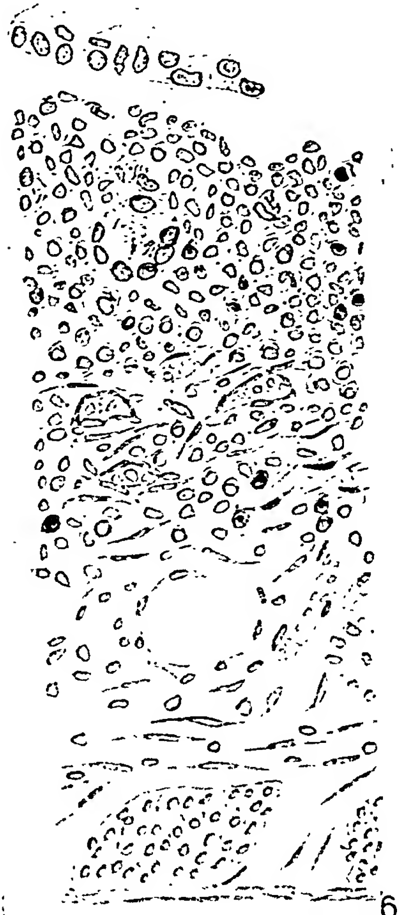

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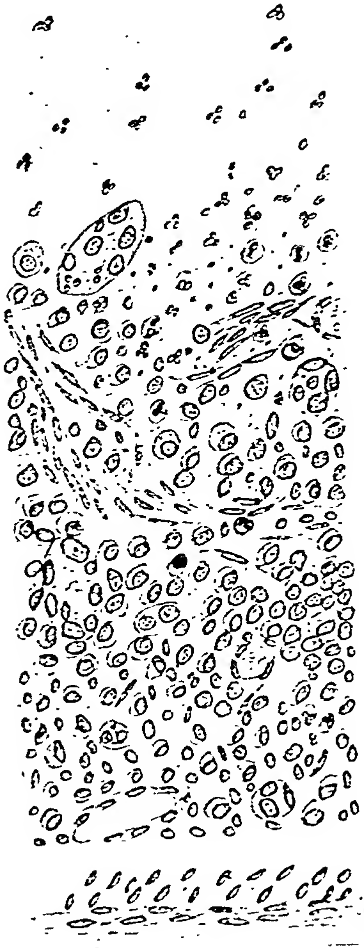

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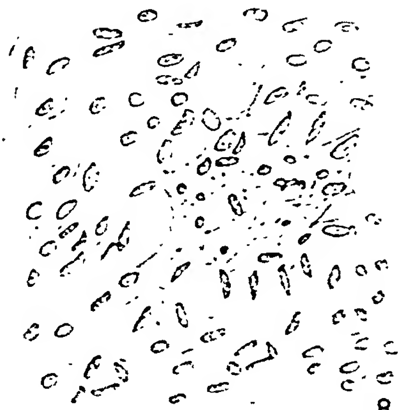

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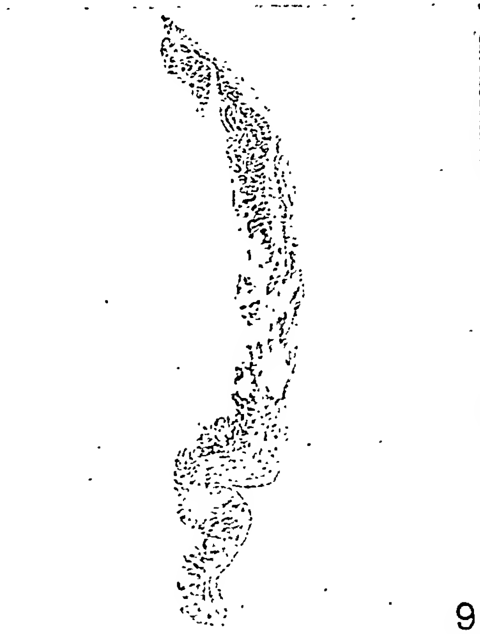
(McConkey and Smith: Vitamin C lack and intestinal tuberculosis)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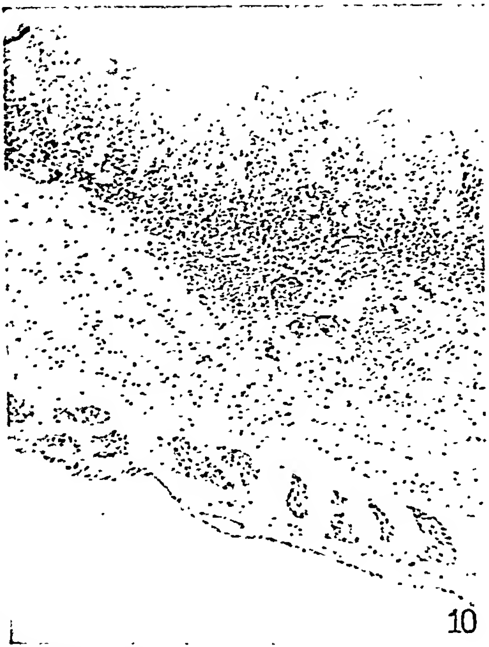
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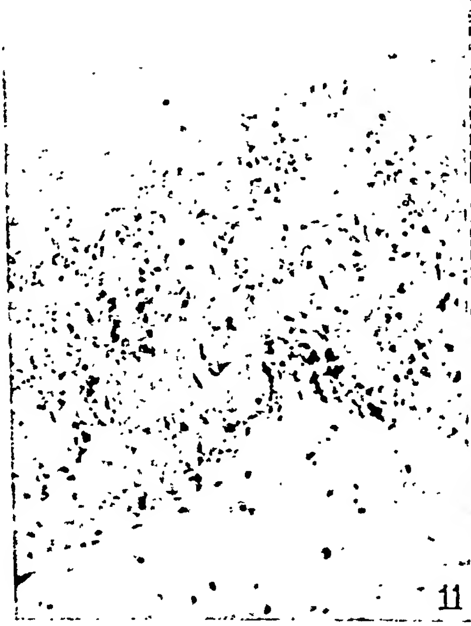
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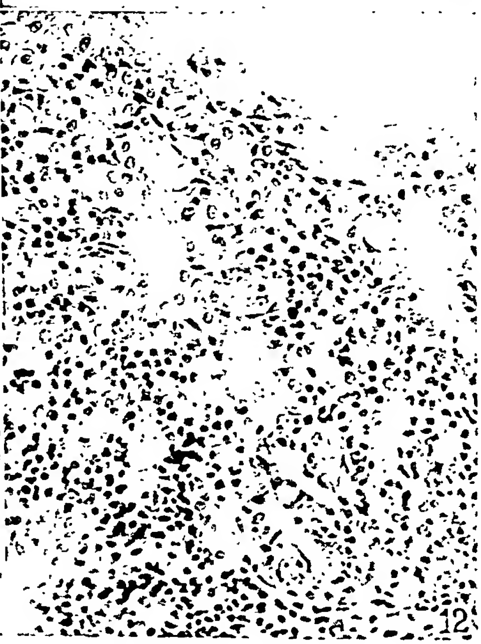
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(McConkey and Smith: Vitamin C lack and intestinal tuberculosis)

A COMPARATIVE STUDY OF RECENTLY ISOLATED HUMAN STRAINS AND A PASSAGE STRAIN OF POLIOMYELITIS VIRUS*

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(Received for publication, June 28, 1933)

Since the early days of the discovery of the virus of poliomyelitis it has been known that quantitative differences in virulence for the monkey exist between recently isolated strains, and strains which have passed through successive monkeys. Only within the last few years has it been clearly demonstrated by the work of Burnet and Macnamara (1) in Australia, that immunologic differences exist between such strains, which might be termed qualitative differences, in that they do not seem to be confined to those of virulence alone.

These investigators made a comparative study of two strains; one a local virus recently isolated from a fatal human case, and the other the Rockefeller Institute strain of so called mixed virus (M. V.). They described three instances in which monkeys contracted a fatal attack of poliomyelitis following the intracerebral inoculation of the M. V. strain despite the fact that some weeks previously the monkeys had sustained a typical attack of poliomyelitis produced by the local strain. The reverse of this experiment was also demonstrated in a single instance, in which a recovered monkey, partially paralyzed by the M. V. strain, was subsequently brought down with complete paralysis by the local virus. Furthermore, they found in neutralization tests that, although pooled convalescent serum would neutralize both strains, a few tests with individual samples of convalescent sera failed to show this parallelism, in that only the local virus was neutralized.

In corroboration of this work Weyer (2) later described differences in the neutralizing values of human convalescent and antiviral horse serum for recently isolated strains and for a monkey passage strain. Flexner (3) has also pointed out that immune sera prepared from "human" and old passage strains of virus

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* The expenses of this investigation were defrayed by several grants from: (*a*) the Fluid Research Fund, Yale University; (*b*) an anonymous donor; and (*c*) the 1932 Philadelphia Poliomyelitis Fund.

exhibit differences in cross-neutralization tests, although old passage strains of the virus preserve their immunizing power in part, at least, against recent strains.

The observations to be reported in this paper are essentially a repetition of the experiments of Burnet and Macnamara, and our results are in accord with their findings. They show that: (*a*) the experimental disease in the monkey induced by our two human strains failed to immunize monkeys against a subsequent reinfection by an old passage strain; and (*b*) that the neutralizing power of human sera for a recently isolated human[1] strain differs from the neutralizing power for an old passage strain.

Methods

Macacus rhesus monkeys, most of them small (weighing 6 pounds or under), were employed in all our experiments. All inoculations were done under ether anesthesia by the intracerebral route.

Strains of Virus Employed.—The old passage strain which we have termed the M strain, was obtained through the kindness of Drs. W. H. Park and E. R. Weyer, from the Bureau of Laboratories, Department of Health, City of New York. It is a mixed strain and so the date of its first isolation or the actual number of monkey passages to which it had been subjected is unknown.

The usual incubation period of the experimental disease produced by this strain was from 3 to 5 days. This disease was heralded by a sharp elevation of temperature to 105° or 106° to be followed within 24 to 36 hours by a sharp drop to subnormal values, widespread paralysis, and usually the death of the animal. A virulence titration done in October, 1931, on material from the cord of a single passage monkey (No. 34) revealed the minimal infecting dose of this material to be greater than 0.5 cc. of a 0.01 per cent suspension of ground monkey cord, and less than 0.5 cc. of a 0.05 per cent suspension. The dose subsequently employed for all neutralization tests with this strain was 0.5 cc. of a 0.1 per cent cord suspension. This technique gave uniform results from October, 1931, to January, 1932. The strain was not used for neutralization tests during the next 14 months, but when later tested (March, 1933), the virulence of material from glycerinated cord No. 34 was found to have fallen. A single passage (Monkey 149) at this time seemed to restore the virulence and, although a titration was not done, the dosage which had been employed with material from Cord 34 was employed with Cord 149.

The recently isolated or human strain which we have termed our W strain was isolated in September, 1931, from the oral washings of a child of 5 years of age on the 1st day of a mild abortive attack of poliomyelitis. The circumstances under

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[1] The term human strain to designate a recently isolated strain has been employed because of its usage in previous articles (2, 3) on this subject.

which the strain was isolated, the technique employed, and the results of the first two passages have been described in a previous publication (4). The results of subsequent passages will be described in a later section of this paper. Material from a single (sixth passage) monkey (No. B-6) was employed for all of our reinoculation and neutralization tests. The usual incubation period of the experimental disease induced by the seventh passage of this strain was 6 days; the febrile period was generally longer than that of the old passage strain, and the development of paralysis only occasionally as severe. The mortality might be roughly placed at 15 per cent, in that out of twenty-seven positive inoculations but one monkey died within 3 days of the onset of the disease and three other monkeys, which developed severe and extensive paralysis and probably would have died, were sacrificed on the 3rd or 4th days of the disease. The histological lesions from this and our other recently isolated human strains failed to show any differences from those noted with the passage strain. The virulence of the sixth passage of the W strain was far lower than that of the M strain. The dose employed in all reinoculation and neutralization tests was 0.5 cc. of a 5 per cent cord suspension, and, as will be subsequently shown in Text-fig. 1, this dose infected fresh monkeys consistently.[2]

A third strain (F) was obtained through the kindness of Dr. Simon Flexner of The Rockefeller Institute for Medical Research. It had been isolated during the summer of 1931 from a fatal case of poliomyelitis and the sample we received represented the eighth passage. A titration of virulence at this time led us to employ the same dosage as that used in our W strain for all neutralization tests. The experimental disease induced by the F strain was quite similar to that produced by the seventh passage of our W strain.

Technique of Neutralization Tests.—In all of the neutralization tests with the M strain the virus-containing material was derived from two monkeys, Nos. 34 and 149. A virulence titration had been done with the former lot of material. In all tests with the W strain the virus was derived from a single monkey, No. B-6, and a series of monkeys had been brought down consistently by the dose employed. With the F strain pooled material from three monkeys was used representing the eighth and ninth passages.

In performing neutralization tests the following technique was employed: A small portion (0.1 to 1.0 gm.) of glycerinated spinal cord was weighed and ground for 6 or 8 minutes in a mortar with sterile sand and 2 to 3 cc. of saline solution. Enough saline was then added to make the percentage of the virus suspension equal to twice that of the dose to be inoculated. This material was centrifuged at moderate speed for 8 to 10 minutes. The opalescent supernatant fluid was then removed with care to avoid the presence of macroscopic particles. Individual tests were set up by mixing equal volumes of the virus suspension and the

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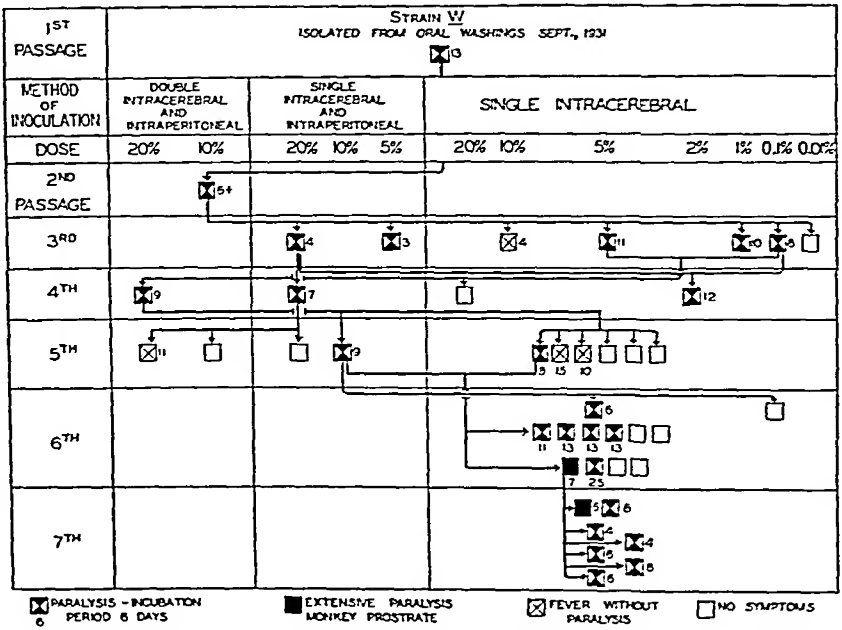
[2] To our knowledge this is the first strain, isolated from the throat, which has been studied intensively through a series of monkey passages.

serum to be tested, using from 0.3 to 0.5 cc. of each. Thus the total volumes varied between 0.6 and 1.0 cc. From one to twenty samples of sera were employed in each experiment but our usual experiment consisted of six or eight samples. Included in each experiment were two control samples of sera: (*a*) the protected control, consisting of a pooled sample of sera from adults recently convalescent from a frank case of poliomyelitis; and (*b*) the unprotected control, which consisted of a sample of normal monkey serum. The serum-virus suspensions were mixed and incubated at 37°C., for 2 hours. A single monkey was inoculated intracerebrally with 0.5 cc. of each serum-virus mixture. Temperatures were taken daily for a period of 4 weeks on all monkeys inoculated with our human (W) strain, on most of the monkeys inoculated with the passage (M) strain, and on a few with the (F) strain. The results of individual tests were expressed by three terms which are, of course, only relative in their meaning: (1) no neutralization (or no protection); (2) partial neutralization; (3) complete neutralization. Sera were judged as having no neutralization when inoculated monkeys came down with the experimental disease within 5 days of the time when the unprotected control came down; partial neutralization when the incubation period proved to be 6 or more days longer than that of the unprotected control; and complete neutralization when the animals failed to come down during the period of observation, which in all instances was 4 weeks. Untoward results were encountered in three out of twenty-one experiments of this type, in all three of which the protected controls developed the experimental disease after a prolonged incubation period. This occurred once with each of our three strains of virus. The results of all tests in these three experiments were discarded and the experiments repeated. It may be worth mentioning, however, that the majority of results of individual tests in the unsatisfactory experiments were in agreement with the results from satisfactory experiments.

In the interpretation of the results of this test it should again be emphasized that in our hands, it has proved to be a rather crude, qualitative test. We seldom attempted to quantitate our results because, owing to the expense involved, the use of several monkeys for each determination was impossible. The degree of accuracy of the test has been difficult to determine. We have had occasion to repeat eighteen individual tests, representing ten different samples of sera, with the W strain and discrepant results were encountered twice, or in about 11 per cent of these repetitions. When discrepant results were encountered at least three tests on the sample of serum were always done and the majority result accepted. Nine tests with the M strain were repeated with a reduplication of results in all but one in which the difference was slight (partial protection with an incubation period of 24 days, as opposed to complete protection).

EXPERIMENTAL

It was our aim in these experiments to employ a strain of poliomyelitis virus as little influenced as possible by monkey passages. This is a lifficult ideal to attain; for, if a strain of virus is to be adequate for use n a series of neutralization tests, it should be of sufficient virulence so hat a single inoculation of at least a 5 per cent cord suspension should onsistently give rise to the experimental disease in the monkey. In either of two freshly isolated strains, W and Rn (4), with which we ave worked was this the case in the first few passages, and considerable difficulty was encountered in rendering one of them adequate for this purpose. As the manner in which a strain of virus, recently isolated from a human case, becomes established in the monkey is pertinent to the problem under discussion, the steps of this process will be given in some detail.



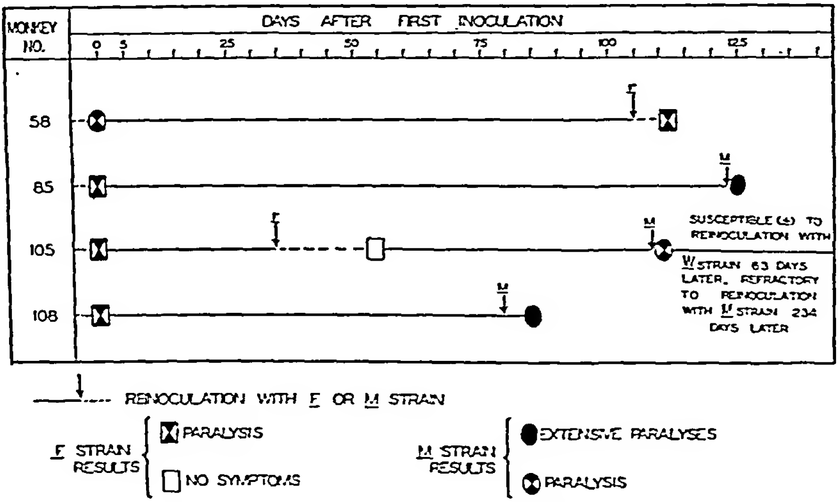
TEXT-FIG. 1. Diagram of the first seven monkey passages to which our human W strain was subjected.

Enhancement of Virulence in the W Strain.—As previously mentioned the circumstances under which this strain was obtained from oral washings, and the results of the early monkey passages have been described in another publication (4). The results of subsequent pasond inoculation failed to give rise to symptoms except in one instance. This was a monkey reinoculated 17 days after the first onset of paralysis; 18 days later the animal again became ill and new paralyses developed. Krauspe (10) reports a similar isolated example of reinfection. This was described as a severe infection following a third reinoculation, administered 4½ months after the first. Presumably the same virus was used for each inoculation but no mention of this fact is made. In any event, although the number of intracerebral reinoculation experiments which appear in the literature are few and briefly described, it would seem as if the successful reinfection of the monkey by an homologous strain was distinctly uncommon.

We have performed thirteen reinoculation experiments, in which homologous strains of the virus were used and daily temperature records were kept over a period of 4 weeks from the second inoculation. With the passage (M) strain opportunities for experiments of this type do not often arise because monkeys infected with the M strain nearly always die. In all of our homologous reinoculation experiments with the M strain the monkeys employed represented examples of the experimental disease which had been modified by methods we shall presently describe. In these reinoculations the same dose was employed as that which had previously infected the monkey, and the intervals between the onset of paralysis and the second inoculation were 20, 20, 52, 60, and 230 days respectively. None of these reinoculated monkeys showed any symptoms such as fever or signs of illness as a result of the second inoculation. With the recently isolated strain (F), two homologous reinoculation experiments were done at intervals of 34 and 146 days respectively, and, with our other recently isolated strain (W), six such experiments were done at intervals of 17, 20, 21, 56, 101, and 111 days respectively. In none of these were any symptoms produced as a result of the second inoculation. This is a small series and certainly does not exclude the possibility that monkeys may occasionally be reinfected with homologous strains during the intervals studied but we believe, as others have often shown, that such instances of reinfection must be uncommon.

Results of our first group of heterologous experiments appear in Text-fig. 2. The first observation recorded in this group is of particular value not only because the outcome was illuminating to us at the time it was performed, but because it represents the only monkey (No. 58) which survived in our series of more than 50 fresh monkeys infected with the M strain. Several other survivors, which will be subsequently described, represent monkeys which had previously been partially immunized.

Monkey 58 was inoculated with a human serum-virus mixture on Dec. 1, 1931. The usual infecting dose for this strain (0.5 cc. of a 0.1 per cent virus suspension in serum) was employed. The human serum in this test possessed some neutralizing properties for the M strain but failed to neutralize it completely. After a prolonged incubation period the animal became ill on Dec. 25, paralysis of both hind legs was noted on Dec. 27. It eventually recovered and there was a slight return of function to the legs. On Apr. 7, 1932, the monkey was reinoculated with 0.5 cc. of a 5 per cent suspension of the F strain. It became ill on Apr. 15 with paralysis of the arms, tremor, and increased weakness of the legs. The animal was sacrificed on Apr. 18 and an examination of the tissues from the central nervous system revealed the presence of both old and fresh lesions of poliomyelitis in the brain and cord.



TEXT-FIG. 2. Four reinoculation experiments with the human F strain and the passage M strain.

The reverse of this experiment is recorded in three instances which also appear in Text-fig. 2, representing Monkeys 85, 105, and 108. These experiments were done early in our work and may not be as satisfactory as some of the others, in that a larger dose of the M strain (ten times the usual infecting dose) was employed to reinfect the monence of this and other variables probably influenced this experiment. Included among them is perhaps that of the susceptibility of individual monkeys which we believe is an appreciable factor more readily manifest in experiments which concern the relatively weak human strain than the passage strain but it is also reflected in the latter. Thus it will be seen in Text-fig. 3 that of the series of twelve monkeys recorded in this group which were initially infected with the *W* strain, two (Nos. 125 and 141) developed relatively mild forms of the experimental disease. These mild forms were characterized by the picture already described in Monkey 105. Generally there was a high and rather prolonged temperature reaction which was not followed by a sharp fall to subnormal values. The animal usually developed tremor and ataxia during the febrile period but made a good recovery and was left perhaps with slight weakness of one or more limbs, which after 3 to 6 weeks was often difficult to detect. The only two mild examples of the experimental disease induced by reinoculation with the *M* strain occurred in these same two monkeys (Nos. 125 and 141) and we have been inclined to attribute this phenomenon to the presence of a hypothetical resistance to the acquired experimental disease in these two monkeys.

Another variable which we have attempted to control and to which reference has already been made is that of the time interval between inoculations. These intervals have been recorded in Text-fig. 3 in a little different manner from that which is usually employed in order that those monkeys which developed mild forms of the disease might be uniformly included in this chart. The interval between inoculations has been numbered from the day of the first onset of fever to the day on which the next inoculation was done. The effect of this interval on the reinoculation result may be best summarized by the statement that all of the failures to reinfect monkeys by a heterologous strain were encountered when the reinoculations were done less than 60 days from the onset of the previous experimental disease. An analysis of the effect of this time interval is also sho Table I. The figures speak for th es.

Comparison of Dif *n Strains by Reinc* *xperi-*
ments.—The attempt made to make a c study
by methods just outli wo human strain ; or of

the early (third to fifth) passages of the W strain as contrasted with the later (sixth) passage of this strain. A few cross-inoculation experiments were done but the results are insignificant. It may be worth recording, however, that the experimental disease induced by a third passage W strain, gave complete protection to reinoculation by the F (eighth and ninth passage) strain in but one of three experiments; partial protection was developed in the other two.

Comparative Results of Serum-Virus Neutralization Tests with the W and M Strains.—As a further means of comparing the properties of human and passage strains, their individual susceptibility to neutralization by different samples of human sera was tested. In spite of the occasional irregularities encountered with this test (see under Methods) the results, which appear in Table II, show that it is not too crude for demonstrating differences in these two strains. Here are listed a series of comparative tests with the W and M strains on blood samples representing fifteen individuals which include seven frank cases with paralysis, one frank case without paralysis, five abortive cases, and two contacts. The cases have been listed in series according to age and it appears that, regardless of the age of the patient, all but one of the recent convalescent samples from frank and abortive cases showed either partial or complete neutralization with the W strain of virus. The exception, J. D., a child 11 years of age, who had sustained

TABLE I

Character of Symptoms Induced by the Inoculation of a Passage Strain in Relation to the Interval from the Onset of a Previous Infection Induced by a Human Strain

	Time interval between inoculation of passage strain (M) and the onset of a previous human strain infection*	No. of monkeys employed	Per cent which developed			
			Extensive paralysis—animal prostrate	Paralysis of one or more limbs	Fever, tremor, ataxia, and slight weakness	No symptoms
	days					
A	More than 70	6	83%	17%	0	0
B	35–70	10	50%	20%	10%	20%
C	20–35	3	0	0	33%	66%

* All of the human strain infections were induced by the W strain with the exception of three in Group A which were induced by the F strain.

paralysis of both legs, failed to show any neutralization in a sample of blood obtained 18 days from the onset of his disease and again 11 months later. An interpretation of the results of the neutralization tests with the W strain on the mild abortive cases and the contacts will be discussed in the following paper (11).

TABLE II

Comparative Neutralization Tests with a Recently Isolated Human Strain and a Passage Strain of Virus

Patient	Character of illness or contact	Age	Recently isolated W strain			Passage M strain		
			Before or during attack or contact	1½ to 10 wks. from onset of attack or contact	11 to 14 mos. later	Before or during attack or contact	1½ to 10 wks. from onset of attack or contact	11 to 14 mos. later
		yrs.						
R. O.	Intimate contact	1	–	–		–	–	
G. M.	Frank case with paralysis	2		+			–	
D. D.	Frank case with paralysis	2½		+			–	
E. D.	Abortive case	4	–	+		–	–*	
F. O.	Mild frank case	4½		+	+		–	–*
A. H.	Frank case with paralysis	4½		+			–	
R. W.	Abortive case	5	–	±		–	–	
A. P.	Frank case with paralysis	6		+	+		–	–
I. O.	Frank case without paralysis	6½	–*	+	+	–*	±	–
M. D.	Abortive case	10	+†	+*		–	±	
B. E.	Frank case with paralysis	11		+			–	
Ev. O.	Abortive case	11	–*	+*	–	+*	+	+
J. D.	Frank case with paralysis	11		–	–		+*	+
Ed. O.	Intimate contact	12	+*	+*	+	+*	+	+
C. O.	Abortive case	13	+†	+*	–	±†	+	+

–, no neutralization. ±, partial neutralization. +, complete neutralization.

* Test repeated, similar result obtained.

† Test repeated, discrepant results obtained; majority or average result accepted.

Quite a different pattern of results is obtained with the M strain. Of the thirty-one tests recorded in this series there is practical agreement with those in the W series in about one-third. With the M strain the presence of neutralization seems to be somewhat an expression of the age of the child. With the older group (6½ to 13 years of age), there is some correlation between the increase of neutralizing power and the presence of either a frank or an abortive attack as shown by the result obtained with I. O., a frank case; and M. D. and C. O., abortive cases. Of the thirteen instances in which complete or partial neutralization was present for the M strain it was present for the W strain in eight; there are five instances in which neutralization was present for the M strain and absent for the W strain; *i.e.*, the first and third samples from Ev. O., both samples from J. D., and the third from C. O. Although the character of the differences between these two strains is unknown, it is also on the basis of these results that we believe that the use of the term qualitative differences is justifiable in a comparative description of the properties of the M and W strains.

DISCUSSION

Experiments in this report, together with previous observations in the literature (1–3), seem to leave little doubt that by the methods we and others have employed, differences can be demonstrated between recently isolated and passage strains of poliomyelitis virus, although there are common immunologic factors between these two strains. The situation recalls those differences which exist between the street virus and the fixed virus of rabies, or perhaps between vaccine virus and smallpox virus. Our own observations may not afford much opportunity for generalizing upon the subject, based as they are on studies of but two so called human strains, and a single passage strain of poliomyelitis virus, and we recognize that there is no exact knowledge as to the stability or the nature of the differences described between these strains. Nevertheless, we are inclined to consider them as qualitative. Furthermore, it should be emphasized that there are no data which define the limits of a human strain. In fact, those strains which we have designated as human do not perhaps really justify this term, for, during the few passages to which they have been exposed, the properties of one of them, W at least, have changed as has been shown in Text-fig. 1. In spite of these limitations, however, the observations do afford some information for which a practical application may be found. Thus it is our belief that in spite of the present difficulties inherent in the technique of the serum neutralization test, such tests with human serum with a recently isolated human strain of the virus, give a better concept of the antiviral properties induced by the human disease than do similar tests performed with passage strains of the virus. With the passage strain some correlation with clinical events was, however, noted in children who were older than 6 years. The correct interpretation of our neutralization tests performed with this passage virus is still a question and it seems to us that much of the data now in the literature concerning the neutralization of poliomyelitis virus by human sera, founded as it has been, largely upon passage strain experiments, is also open to some question in so far as giving complete or accurate information which has to do with the anti-human poliomyelitis virus properties of the blood.

SUMMARY

1. Confirmation of the qualitative differences which exist between so called human and passage strains of poliomyelitis virus has been established by the following observations.

(*a*) The experimental disease induced by two human strains usually failed to protect monkeys against a subsequent infection by a passage strain, and in the few instances in which the reverse experiment could be tried a similar lack of protection was observed.

(*b*) In some human sera the neutralizing power for a human strain differed qualitatively from the neutralizing power for a passage strain.

2. The time interval between the intracerebral inoculation of heterologous strains has been found to be an important factor bearing upon the results of the reinoculation experiments reported. Within the intervals used, the greater the period between the original infection and the reinoculation with a heterologous strain, the less was the degree of cross-immunity observed.

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NEUTRALIZING ANTIBODIES IN ABORTIVE POLIOMYELITIS*

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INTRODUCTION

In a previous paper (1) we have described the isolation of the virus of poliomyelitis from the throats of two patients, each of whom had developed a brief minor illness during an epidemic of poliomyelitis. This finding together with the experience of many previous observers, and our own field observations (2, 3), have led us to believe that during an epidemic of poliomyelitis many brief illnesses, which present symptoms similar to those of incipient frank poliomyelitis, probably are mild, or abortive forms of the disease. The significance of these abortive forms lies in their frequency, for in observations drawn from two epidemics (the New England epidemic of 1931, and the Pennsylvania epidemic of 1932), they were found to outnumber the frank cases by from four to six times (3). Consequently, within this broader concept of the disease lies a possible explanation of the manner in which many adults acquire immunity to poliomyelitis and it is with a single phase of the latter question that this communication will be concerned, in that it will deal with the detection of neutralizing antibodies for poliomyelitis virus in the blood of individuals who have sustained these mild abortive attacks.

At the onset an explanation is required of our usage of the term "abortive poliomyelitis," for it has been employed differently by different observers. Under this head some have listed (*a*) frank cases of poliomyelitis in which signs of meningitic or myelitic involvement

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* The expenses of this investigation were defrayed by several grants from: (*a*) the Fluid Research Fund, Yale University; (*b*) an anonymous donor; and (*c*) the 1932 Philadelphia Poliomyelitis Fund.

were present, but in which paralysis did not develop; others have listed, either together with these non-paralytic cases or separately, a syndrome (*b*) which presented only the signs of an acute general infection. These different criteria together with our own, have been discussed in one of our previous papers (2). It may suffice to say, however, that our usage of the term abortive poliomyelitis has been applied only to the latter syndrome (*b*), and as such it conforms in part, at least, to that of Wickman to whom the term was originally due. The clinical recognition of these abortive cases is primarily dependent upon their occurrence during an epidemic of poliomyelitis. The clinical picture is that of an acute febrile episode usually of 1 to 4 days' duration, which is generally characterized by such symptoms as vomiting, headache, sore throat, pain in the back and limbs, and listlessness. It conforms closely to that of the first phase of the so called "dromedary" form of the frank disease, and according to Draper (4), is identical with it. We believe that this clinical picture is not as vague as was at one time supposed, even though unexplained fever is still perhaps its major feature, and even though there are no practical tests as yet available for its definite diagnosis. There are, however, certain measures which may be applied for its identification; one of them is the isolation of the virus to which we have already referred (1). Another is the determination of the development of neutralizing antibodies in the sera of individuals who have sustained an attack. Our present study will describe our methods of employing this second procedure without an attempt to interpret the findings in the broader sense of host resistance to poliomyelitis.

The literature dealing with previous attempts to demonstrate neutralizing antibodies for the virus of poliomyelitis from patients who had sustained abortive attacks, reveals somewhat variable results. Soon after the discovery that these neutralizing antibodies could be demonstrated in the serum of patients who had sustained a frank attack of the disease, Netter and Levaditi (5) reported a single instance in which similar antibodies were found in the serum of a child who had sustained an abortive attack. They describe two children in one family—Emile and Henriette. On Jan. 14, 1910, Emile, aged 6 years, developed a frank attack of poliomyelitis with subsequent paralysis. About 10 days previously, Henriette, whose age is not given, had been sickly and cross, would not eat, hesitated to walk, and complained of tenderness in the limbs. A lumbar puncture was not reported in this child. At the end of 3 weeks the child had completely recovered her health.

Samples of blood were obtained about 10 weeks (exact time not stated) after the onset of both illnesses. The serum from the frank and the abortive case neutralized the virus. The character of the strain of virus used is not specified. In their neutralization tests equal parts of serum and virus suspension were employed which, unless otherwise stated, is the technique followed in all subsequent experiments by other authors listed in this review.

A year later Anderson and Frost (6) made a more extensive study of the neutralization test in a series of ten cases which they felt were probably examples of abortive poliomyelitis of the Wickman type. Seven of these ten abortive cases represented adults who were from 22 to 40 years of age. Blood samples were collected from 1 to 4 months after their acute illness. A passage strain of the virus was employed for their neutralization tests which were set up as follows: 0.5 cc. of a 5 per cent virus suspension, 0.5 cc. of serum to be tested, and 0.1 cc. of fresh normal serum. The first series of neutralization tests showed that sera from the seven adult members of the series neutralized the virus, whereas sera from the three juvenile members aged 16, 10, and 7, did not. A repetition of the tests on the sera of the three juveniles in which a weaker (1 per cent) suspension of virus was employed, showed neutralization to be still absent from the sera of the patient aged 16, but to be present in the two younger children. These experiments have been transcribed in some detail because we believe such details to be important in the light of their present day interpretation. It is not surprising that tests from all of the seven adults should neutralize the virus. Furthermore, it is questionable whether the positive results obtained in any of their tests are significant, for as has been pointed out by Shaughnessy, Harmon, and Gordon (7) the results may have been influenced by the addition of normal human serum to activate the serum virus mixtures. Nevertheless, the negative neutralization tests on a single child who had sustained an abortive attack should be noted, even though they were performed by a rather unusual method.

At about this time Müller also reported a single instance of a positive neutralization test in a child who had sustained an abortive attack. Details of the test or the time interval after the disease at which the sample was obtained, are not, given (8). Peabody, Draper, and Dochez (9), using a 1:10 virus-serum mixture, reported a positive neutralization test in the serum from one of three suspected cases, although according to the diagnostic criteria employed by these observers, the case with neutralization would today be classified not as a true abortive case but as a non-paralytic frank case, in that it showed a pleocytosis in the spinal fluid.

During the subsequent 20 years, in spite of the fact that knowledge of technical details of the neutralization test increased, and that considerable data were collected upon the incidence of normal individuals in various populations and age groups whose sera gave positive tests, little or no work seems to have been done upon the relation of this antibody content to the abortive case. It became evident during this period that sera from the majority of supposedly normal adult individuals contained neutralizing antibodies for passage strains of the virus. Many of the observations on this point have been summarized by the recent publication of the International Committee for the Study of Infantile Paralysis (10) to which the reader may be referred. It also appeared during this period that certain discrepancies in results were obtained with the neutralization test on both convalescent and normal sera by different observers. Some of these results have been summarized and compared by Howit (11), who assigns her own low percentage of positive neutralization tests on convalescent sera from frank cases, among other reasons, "perhaps to the fact that the adapted monkey strain virus is not regularly neutralized by the human antiviral substances, especially if the latter are in low concentration in the blood."

However, in 1931 the abortive case and its relation to the presence of antiviral substances in the blood again began to receive attention (12). A year previously, Fairbrother and Brown (13) obtained samples of sera from a series of juvenile inmates of a boarding school which had sustained an outbreak of poliomyelitis. 6 months after the outbreak, sera were obtained from nine of the inmates whose ages ranged from 8 to 13 years. One of them had sustained an abortive attack, four had sustained frank attacks of bulbar poliomyelitis, two had been in close contact with these cases, and two had been in remote contact. Sera from two children who had arrived at the school after the epidemic were included as controls. Neutralization tests upon this group showed that all of the sera except the two controls possessed antiviral substances for a virulent passage strain of the virus. The authors concluded that the positive neutralization tests observed in the contacts supported the view that the immunity of adults is most probably due to subclinical infections.

A year later Kramer and Aycock (12) reported an experiment of similar nature. The circumstances under which their experiment was performed were as follows: In October, 1930, five cases of poliomyelitis occurred in a small Massachusetts town. Residence was established in the town with the appearance of the first case and the population was canvassed. The histories of all illnesses which occurred during the epidemic were collected, and approximately 50 mild illnesses, which conformed in their symptomatology to abortive poliomyelitis of the Wickman type, were found. 5 months later sera were obtained from about half of this group of 50 children. As controls, sera were also obtained from similar age groups of (*a*) local children, who had not passed through any recognizable illness, and (*b*) children from an adjacent town where no poliomyelitis had occurred. The results of the neutralization tests in these three groups of children proved to be almost identical; that is, the sera from 54 to 60 per cent of the school children under 15 years of age in all of the three groups tested failed to show the presence of neutralization, or in the words of the authors, "The expectation of a high rate of immunity in those having passed through the mild illness did not materialize." Another aspect of this problem which deals with so called subclinical or latent immunization occurring in association with an epidemic was subsequently tested by Kramer (14). From a group of twenty-three persons who had been in intimate or prolonged contact with a patient with poliomyelitis, sera were obtained within a few days following termination of the contact, and in twelve of these samples neutralization tests were negative. Six of these twelve cases were available for retesting after 5 months and at this time the neutralization tests were positive. Sera were also obtained from another group of fifteen persons several days following casual and indirect contact; eight failed to neutralize and of these, six were available for retesting 3½ months later. Here the late tests were found to be negative. He concluded that, "The evidence presented indicates that immunity may follow exposure to the virus without evidence of disease."

In résumé, therefore, the previously reported attempts to detect the presence of neutralizing antibodies for the virus following abortive attacks of poliomyelitis have yielded some conflicting results and widely different interpretations. The presence of an acceptable positive neutralization test following an abortive attack has been reported in three single instances by three groups of observers (5, 8, 13), whereas a single negative instance has been reported by another group (6); and in the large number of possible mild abortive cases tested by Kramer and Aycock (12), no appreciable increase in neutralization in the whole group was noted. What renders the interpretation of these findings still more difficult is the impression that neutralizing antibodies may occur with considerable regularity (13), or frequently (14), in the blood of contacts who have passed through an epidemic without having sustained a frank or an abortive attack of the disease.

Several features should be pointed out which, according to data presented in this and our previous paper (15), probably influence the interpretation of the results of these tests. These are: (*a*) all of the tests which we have transcribed from the literature in which either the presence or absence of neutralization following an abortive attack of poliomyelitis is described, have been based on single convalescent samples of serum; (*b*) those in which negative results were reported were obtained 4 or 5 months after the illness, and the negative results have been reported with the implication that the absence of neutralizing antibodies at that time suggested that such antibodies had not been previously present; and (*c*) passage strains of the virus were employed in testing the neutralization of the sera from all of the suspected abortive cases and from contacts, except in two of the earlier publications in which the character of the strains is not specified (5, 7).

Methods

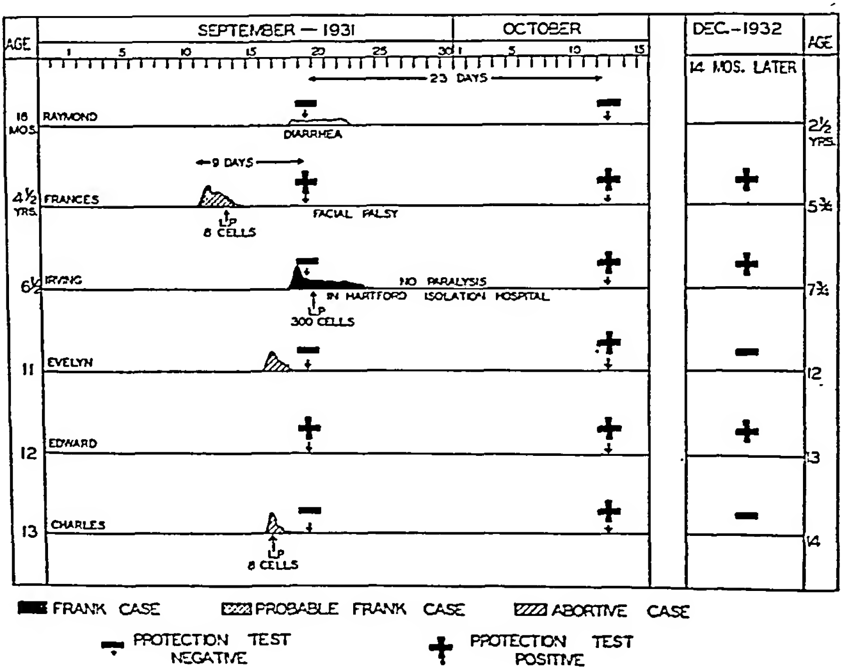
It has been our aim in this work to obtain serial samples of blood from individual patients (*a*) before or during an attack of mild abortive poliomyelitis, (*b*) during convalescence, and (*c*) several months later; and to compare the virus-neutralizing properties of these serial samples from each patient.

For the neutralization tests both human and passage strains of virus were employed. The reasons for laying emphasis upon the use of a human strain for these tests are given in the previous paper (15), together with a critical review of our experiences with the test, the technique and principles which we have employed, and a comparative series of results with a human and passage strain in frank cases. Primarily, certain points should receive emphasis, particularly as we believe that the attempt is often made to obtain more information from the neutralization test in this disease than it actually at present affords. In our hands it has proved but a rough qualitative estimate of an unknown amount of neutralizing antibody for a given strain of virus, for we have not tried to quantitate the results. What relationship this unknown level of neutralizing antibody for various strains of the virus bears to questions of clinical immunity in poliomyelitis is also unknown. We believe that the most tangible information to be gained from the qualitative test is that afforded by testing serial samples of serum from the same individual in the same experiment. If serial tests thus performed show different results, then one can say that the neutralizing antibody content has changed in a given direction provided the tests were above reproach, but here again, and especially in using a human strain of virus, and sera in which the antiviral content is close to the borderline of neutralization, the susceptibility of individual monkeys or perhaps other unknown factors have occasionally given rise to discrepant results (15).

EXPERIMENTAL

The number of available individuals upon whom it was possible to obtain samples of blood before, during, and after an attack of mild abortive poliomyelitis was necessarily small and the results shown in this paper represent two small groups of individuals from each of whom two or three samples were obtained. All of the patients studied were seen in the vicinity of New Haven during the 1931 epidemic.

The first group of individuals which have been studied intensively, represent a family composed of six children in which an epidemic of poliomyelitis occurred. A schematic diagram of this family (Or.) has been published in one of our previous articles which describes an unsuccessful attempt to isolate the virus of poliomyelitis from the throats of these particular children (1). Five of the six children in this family became ill between Sept. 10 and 16, 1931, and in four of them the illness was initiated with practically the same symptoms; namely, fever, headache, vomiting, and pain in the neck, back, or limbs. One of these illnesses (that in the child Irving) turned out to be a frank case of poliomyelitis. Another child, Frances, developed what was probably a frank case in which the diagnosis of nervous involvement rested upon the fact that she sustained a residual facial paralysis. In two other children the symptoms were typical of mild abortive poliomyelitis and it may be a little more than chance that these two abortive cases occurred in older members of the family than did the two frank cases, for as we have recently emphasized, in this particular epidemic at least, the abortive cases tended to appear in an older age group than did the frank cases (3). The fifth illness occurred in an infant of 16 months and merely consisted of diarrhea of 4 days' duration.



TEXT-FIG. 1. Diagram of Family Or., in which are shown various illnesses designated by solid and shaded areas and also the results of the neutralization (or protection) tests taken during and after these illnesses.

We have in this family two examples of mild abortive poliomyelitis representative of the syndrome which we wish to test; two positive controls, in that they represent frank cases; and two contact, or negative controls which represent either no illness at all or an illness (brief diarrhea of unexplained origin) which does not conform to the clinical picture of abortive poliomyelitis.

A series of blood samples were obtained from all of the children in this family on Sept. 19, 1931, which was during the period when most of the children were or had been recently ill. A second series was obtained three weeks later on Oct. 12. A third series, obtained 9 months later unfortunately was lost. A fourth series was obtained 14 months later from all but the youngest child, who, during the interim, had been placed in a foster home and was not accessible.

The results of the first tests with our human or W strain in its seventh passage, which appear in Text-fig. 1, represent two individual experiments embracing all but one of the seventeen samples of sera which were tested. It was our object in these two experiments to include all three tests on the same patient in the same experiment thus controlling the amount of virus used in serial tests. In both frank cases (the positive controls) neutralization was present 3 weeks after the illness and in one of them 9 days after the onset of symptoms, and in both of the late samples (taken 14 months later) neutralization was again found to be present. In the two abortive cases neutralization, which had been absent 3 days from the onset of symptoms, had appeared after 3 weeks but had again sunk below the quantitative level of the test 14 months later.[1] Of the two contact controls the youngest child failed to show neutralization in either sample and the older child (Edward) was found to have neutralizing antibodies in all three specimens. The tests on neither of the two contacts can therefore be considered as satisfactory from the standpoint of giving information about the latent development of neutralizing antibodies. The apparent eventual decline of neutralizing antibodies revealed by the two abortive cases is in keeping with that seen in many infectious diseases but it is somewhat at variance with the usual concept held in poliomyelitis; namely, that once a given level of neutralizing substances appears in the blood they persist. However, a reasonable criticism of the orthodox view is that most of the neutralization tests upon which it has been based have been done with passage strains of the virus.

The second group of mild abortive cases in which serial blood sam-

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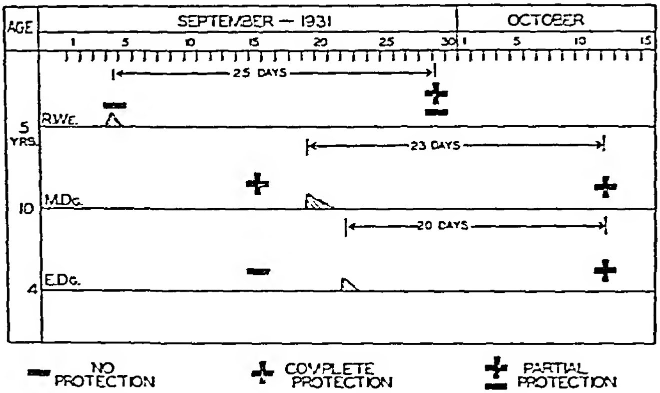
[1] As will be subsequently seen, the first sample of serum from Charles was probably close to the border line between a positive and negative test. Repetitions of this test were positive and represent the single apparent discrepancy in the results shown in Table I, as compared with the results in Text-figs. 1 and 2.

ples were obtained (Text-fig. 2), is composed of three children who were members of a small community in Madison, Conn., which sustained an extensive epidemic of poliomyelitis during the summer of 1931. Details of this epidemic have been described in another paper (1), in which a diagram is shown giving the time relationships between the twenty-five abortive and the four frank attacks of poliomyelitis which occurred among forty-one children in this community.

All of the three children studied in this report had presumably been exposed to poliomyelitis in this community on several occasions during a period of 35 to 40 days prior to the time at which they developed an abortive attack. From the throat of one (R. We.) of these three children, the virus of poliomyelitis had been isolated on the day of his illness. A blood sample was obtained at this time, Sept. 4, and a second (convalescent) sample was obtained on Sept. 29. The two other children in the group of three from the Madison community represented two members of the same family, in whom we were fortunate to obtain blood samples a few days prior to the time when they contracted mild abortive attacks of the disease. These first samples were obtained on Sept. 15. One of the children, M. Dg., aged 10, developed an abortive attack on Sept. 19; the other child, E. Dg., aged 4, an abortive attack on Sept. 22. A second (convalescent) sample was obtained on Oct. 12, 20 and 23 days after the onset of these illnesses.



TEXT-FIG. 2. Three cases of abortive poliomyelitis in which neutralization (or protection) tests were done on convalescent and earlier samples of serum.

The results of the tests with the human or W strain appear in Text-fig. 2. They represent two neutralization experiments in which both samples of blood from R. We., were included in the first experiment and both samples from the other two children in the second. In two of these children an increase of antiviral substances was found subsequent to the abortive attack; in the other child, M. Dg., neutralization was present 9 days before she developed an abortive attack, and also in the convalescent specimen. Whether or not there was a change in the neutralizing antibody content of the convalescent sample from M. Dg. as opposed to the pre-illness sample is not determined by these tests, but, as will be subsequently shown with the passage or M strain, a slight increase was demonstrated in the second sample from this child. The presence of neutralizing antibodies in the first pre-illness sample from M. Dg. tested in this experiment is difficult to explain, and although the result has been modified somewhat by subsequently repeated tests with the W strain (two tests have been positive and one negative) it nevertheless shows us that a level of neutralizing antibodies, which was probably close to the borderline of a positive or negative test, did exist in this child prior to the time in which she became ill with abortive poliomyelitis.

Comparison of Neutralization Tests with the Human (W) Strain and the Passage (M) Strain

In Table I is shown a comparative series of results with two strains, our human (W) and a passage (M) strain. The manner in which these results have been recorded in the table deviates somewhat from the manner in which they appear in Text-figs. 1 and 2, in that some of the results marked by an asterisk or a dagger represent not a single experiment but the average of several experiments and the majority results have been recorded. This gives rise to a single apparent discrepancy; namely, the result of the test with the W strain on the first sample from Charles Or. in which neutralization was absent in the first experiment (recorded in Text-fig. 1) and present in two subsequent experiments.

Differences between the pattern of results obtained with the two strains are quite apparent and have been discussed in the previous publication (15), in which the influence exerted by the age of the patient upon the result of tests performed with the passage strain has been pointed out. Those performed with the M strain upon these children who were under 10 years of age show no demonstrable effect which might have been induced by an abortive attack of poliomyelitis, but on the other hand, in two of the older children (10 and 13 years of age), there is an apparent increase in neutralizing power associated with the abortive attacks. It is also important to note that, although in these same two cases of abortive poliomyelitis (Charles Or. and M. Dg.) no consistent increase in antiviral content could be demonstrated in the early convalescent sample of sera with the W strain, in both of these cases a slight increase was demonstrable with the M strain.

TABLE I

Comparative Neutralization Tests with a Human Strain (W) and a Passage Strain (M) on Five Cases of Abortive Poliomyelitis

Patient	Age	Human (W) strain			Passage (M) strain		
		Before or during attack	3 wks. later	14 mos. later	Before or during attack	3 wks. later	14 mos. later
	yrs.						
E. Dg.	4	−	+		−	−	
R. We.	5	−	±		−	−	
M. Dg.	10	+*	+†		−	±	
Evelyn Or.	11	−†	+†	−	+†	+	+
Charles Or.	13	+*	+†	−	±*	+	+

−, no neutralization. ±, partial neutralization. +, complete neutralization.

* Test repeated, discrepant results obtained, majority or average result accepted.

† Test repeated, similar result obtained.

The significance of this finding is not apparent. One cannot say that the dividing line between a positive and negative test with the M strain represents a quantitative level of the same neutralizing antibody which is merely higher than that recorded by the W strain, for, as previously emphasized (15), there are qualitative differences between these two strains. Nevertheless, in spite of the relative inaccuracy of the tests employed, and of our inability to interpret the results adequately, we believe that information of some clinical or immunologic significance can be obtained from each set of tests, and that the data presented in Table I answer the question in the affirmative as to whether or not neutralizing antibodies appear in the blood shortly after a mild abortive attack.

DISCUSSION

Our results show that in at least three out of the five cases of mild abortive poliomyelitis in which serial samples of blood were tested with a human strain of virus there was a greater neutralizing capacity in the convalescent (3 weeks) sample than existed in an early sample obtained during or before this period of acute illness and that the two other cases, tested with a passage strain, also showed a similar although slight increase. It is conceivable that these findings bear no relationship to the acute illness through which the patients had recently passed, and may rather be an expression of certain physiological fluctuations in endocrine balance, which Jungeblut and Engle have recently described (16), but we think this unlikely in this particular instance.

Furthermore, there are certain features of this work which appear to be a little at variance with current opinions about the average rate of the production of neutralizing antibodies in the blood of individuals convalescing from frank and abortive cases of the disease and the subsequent persistance of these antibodies. It is not surprising that such is the case because in most previous experiments dealing with this problem the work has been done with a passage strain of the virus. Our observations with the human strain record the fact that when neutralizing antibodies appear in association with an abortive or frank attack they generally appear rapidly; that is, within 3 weeks and perhaps (Charles Or.) within a few days. In the frank cases tested about a year later these antibodies were found to persist, whereas in the two abortive cases similarly tested, the antibodies had again fallen below the quantitative level of the test. The suggestion is that the most severe cases, or at least those in which demonstrable involvement of the central nervous system occurred, developed a higher antibody content than did the mild abortive cases although the data on this point are too limited to warrant discussion. It is possible, although we have no records of its occurrence, that individuals may frequently sustain more than one abortive attack; that there may be a cumulative building up of neutralizing antibody content of the blood by this, and perhaps other influences (17), to account for the relatively higher titers usually recorded in adult samples of blood, but we would call attention to the fact that here again there is little knowledge of the antibody content of the blood for human strains of poliomyelitis virus in various adult populations, because tests with human strains on an appreciable number of adult individuals have not to our knowledge been done.

In conclusion, as stated in the introduction of this paper, it has not been our object to attempt to generalize with regard to our findings in terms of resistance or general immunity to poliomyelitis. The reasons for this are that we regard the presence of neutralizing antibodies in the blood, at least as measured by the methods recorded in this paper, as but one phase of immunity to poliomyelitis. The major significance of our results rests not in an explanation of mass immunity to poliomyelitis, but that they further confirm a view, held previously by many observers, that certain characteristic types of minor illnesses which accompany an epidemic of poliomyelitis represent mild cases of the disease.

CONCLUSION

The neutralizing antibody content for poliomyelitis virus has been tested with both a human and a passage strain of the virus in serial samples of sera from five mild cases of abortive poliomyelitis, and an increase in this antibody content has been demonstrated in convalescent samples obtained within 4 weeks of the acute illness.

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FURTHER STUDIES ON THE HYPOPHYSEAL SUBSTANCE GIVING INCREASED GONADOTROPIC EFFECTS WHEN COMBINED WITH PROLAN*

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In a previous study (1) of the greatly increased gonadotropic effect of prolan when it is combined with some component of the anterior hypophysis, it was pointed out that the hypophyseal substance in question could be neither the growth nor gonad-stimulating hormone. Indeed, at that time it seemed that this substance had no physiological effect of its own, but was dependent upon its combination with prolan for the exhibition of activity. It can now be shown that the substance (the so called synergic factor) exhibits peculiar gonadotropic effects which distinguish it from any substance hitherto isolated from the hypophysis. When it is administered alone (*i.e.* uncombined with prolan) in three daily injections with autopsy of the animals at the end of 96 hours, it has produced little or no enlargement of the ovaries; yet sacrifice of the experimental animals at 36, 60 and 84 hours (Chart 1) shows that definite gonadotropic properties are possessed by this material, evidenced by slightly increased ovary weights as early as the 36th hour after the beginning of dosage, an effect earlier than that secured by some of the most potent gonadotropic preparations known. For example, the remarkable gonad-stimulating hormone from pregnant mare's serum produces a slower initial development of the ovary,

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which increases rapidly after about 72 hours so that the maximum effect from three daily doses is produced in 96–120 hours. Prolan is similar in its insignificant early effect on the ovary but the ovary weights plateau after 72 hours. These types of ovarian development are shown graphically in Chart 2 (*a*) and the comparison of prolan,

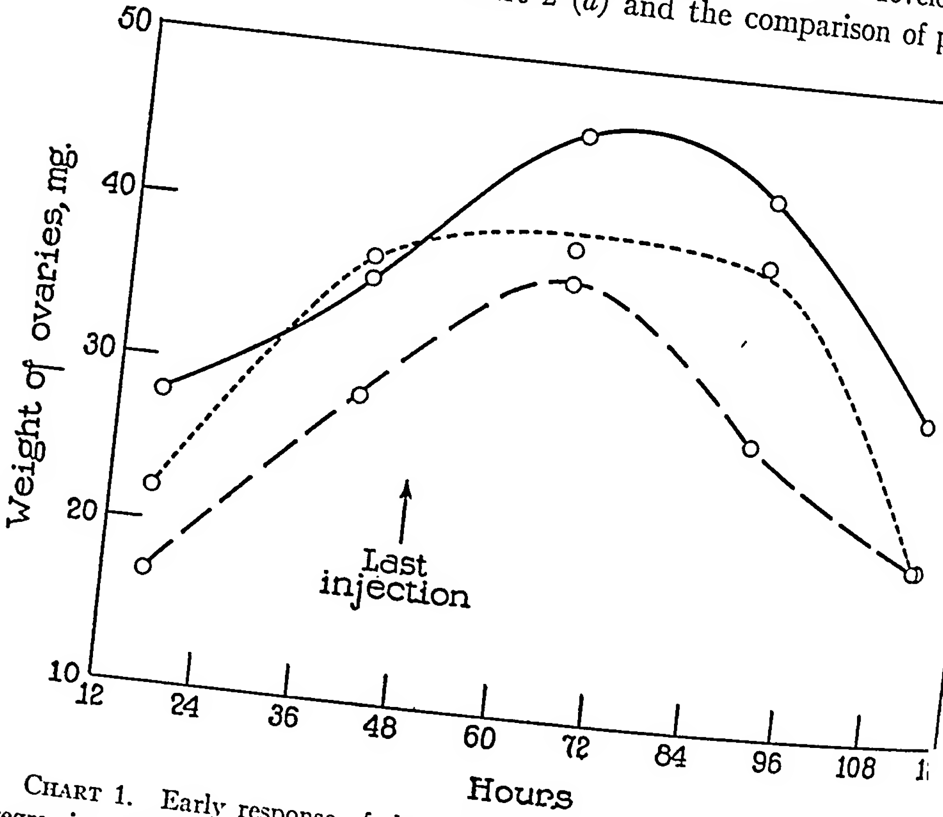

CHART 1. Early response of the ovary to the synergistic factor followed by regression within 96–120 hours. The preparations indicated have been subjected to different treatments:

——— Glacial acetic acid (R3521).

- - - - - - - Isoelectric precipitation (supernatant) (R3523).

— — — Trypsin and erepsin digestion (R3522).

synergic factor, synergic factor in combination with prolan, and pregnant mare's serum may be made.

When the injections are continued for a total of 6 days (instead of the usual three day injections with autopsy at 96 hours) the development produced in the ovaries by the synergic factor does not regress but is maintained. Chart 2 (*b*) shows the ovarian weights produced by this method of injection using the same gonad-stimulating substances as before. In the cases where the synergic substance was injected for 6 days (twice daily, as in Chart 2 (*a*)), the ovary weights do not decrease after 72–84 hours as in the case of the three injections.

The increased ovary weights are at first not paralleled by clear morphological expression. Even though increase in ovarian weight is noted before 36 hours, the ovary may show only a slightly increased vascularity. An increase in the number of medium sized follicles is, however, usually noted by 24–48 hours. Some ovaries show no further development even by 113 hours. This is true for example of the synergic fraction prepared by trypsin and erepsin digestion

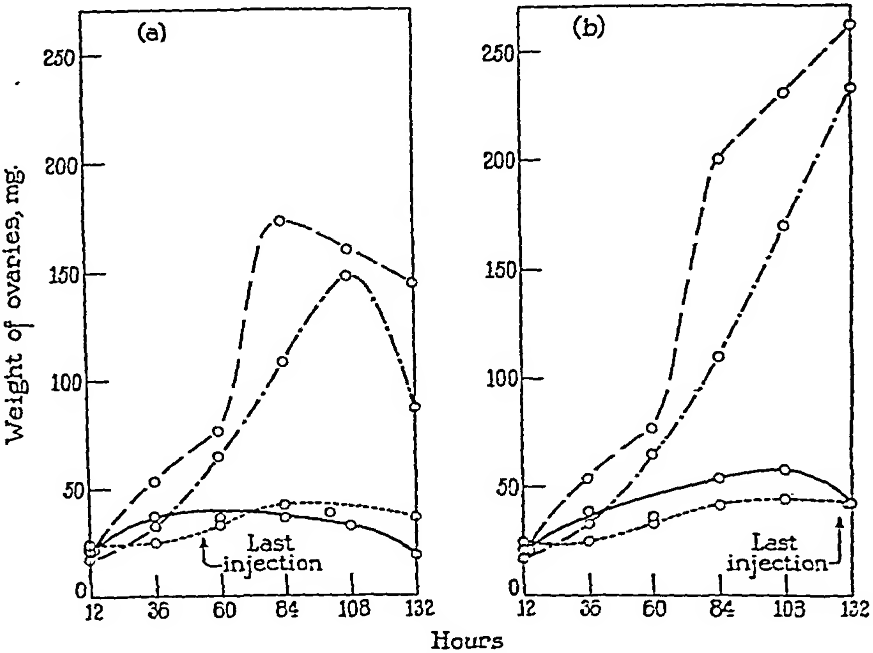

CHART 2. Response of the ovary to various gonadotropic hormones with (*a*) injection for 3 days (twice daily) and (*b*) injection continued for 6 days.

———— Synergic factor (R2721).
- - - - - - - - Prolan.
— — — Synergic factor in combination with prolan.
—·—·— Pregnant mare serum.

(R3522) shown in Chart 1. However, the ovaries of animals injected with the synergic factor more frequently show by 40–84 hours at least a few intermediate bodies or small and medium corpora. Ovulation has never been noted. Uterine development, vascularization and increase in size, is often observed by 24–48 hours.

Non-Identity of the Synergic Factor with Other Hypophyseal Substances

In 1931 Fevold, Hisaw and Leonard of Wisconsin (2) reported the fractionation of pyridine extracts of the entire hypophysis into follicle-stimulating and luteinizing components, the latter fraction without detectable action on the ovary when administered alone; but when combined with the former, it increased markedly its action. Indeed, the whole efficacy of unfractionated pyridine extracts was again secured by recombination of the two fractions. The recombination phenomenon resembles so strikingly what we have described as the activation phenomenon resulting from the addition of prolan to some hypophyseal substance, that an examination of the two procedures for possible underlying identity is important. Furthermore, Leonard (3) has shown that the follicle-stimulating fraction of Fevold, Hisaw and Leonard (2) gave augmentation when combined with prolan. Hisaw and his collaborators have also pointed out (4) that prolan and their luteinizing fraction behave similarly in augmenting the action of the follicle-stimulating fraction. In comparing the properties of the hypophyseal synergic factor prepared in this laboratory with the properties ascribed to the follicle-stimulating fraction (2, 5) several differences became apparent. Firstly, the synergic factor given in increasing doses, though producing definite ovarian development in 96 hours, does not give the large ovaries described for the follicle-stimulating fraction. In Table I, it is seen that a dose of the synergic principle 100 times that necessary to give activation (the minimal effective dose) produces only moderate ovarian development.

Secondly, the synergic factor which is very potent in its ability to augment the action of prolan gives only moderate ovary weights (Table II) when combined with the luteinizing fraction. However, the follicle-stimulating fraction is augmented by both the luteinizing fraction and prolan (4), producing large ovary weight as well as high percentage activation.

Most fractions of the synergic factor so far obtained give some luteinization of the ovaries in 96 hours although uniform enlargement of the follicles to medium sized structures is the chief effect. Whether the luteinization is an inherent property of the substance, is due to contamination by the luteinizing fraction or is due to the animal's own hypophysis cannot be said at present.

TABLE I

Gonad-Stimulating Action of Large Doses of the Synergic Factor

Synergic factor			Prolan		Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Aqueous alcohol (pig) R2586	0.27	18			54	25
	0.54	20	13.6	43	102	126
	1.36	25			129	168
Aqueous alcohol (pig) R3524	1.36	22			107	181
	2.73	28			130	195
	6.8	27	13.6	34	128	197
	13.6	26			118	181
	27.3	36			131	162
	54.5	67			141	72

TABLE II

Combination of Luteinizing Fraction with the Synergic Factor

Synergic factor			Luteinizing fraction		Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Aqueous alcohol (pig) R2834,	2.7	26	13.6	22	41	36
R3160	1.36	21	27.3	23	94	261
Aqueous alcohol (sheep), supernatant after isoelectric precipitation R3330	0.27	17	27.3	23	64	178
Alkaline extract (sheep), trypsin and erepsin digested R3331	1.36	25	27.3	23	80	166
Acetic acid powder (sheep) R3332	13.6	19	27.3	23	54	125

These facts clearly indicate that the synergic factor, prepared in this laboratory, cannot be identified with either the luteinizing or follicle-stimulating fractions and is actually a third gonadotropic substance from the pituitary. Indeed, it would appear that the follicle-stimulating fraction (2, 5) is a mixture of the synergic factor herein described, with what has been designated as the follicle-stimulating substance. There is no question as to the non-identity of prolan and the luteinizing fraction.

It would appear, then, that we are not justified in identifying the above mentioned recombination effects with our activation experiments, that indeed a comparison of the two experiences cannot as yet be instituted, although future research may disclose an underlying reason why the combinations in question should necessarily increase in each instance the gonadotropic effect.

Antagonism of Gonad-Stimulating Effects by Some Hypophyseal Component

Some years ago it was shown that the normal gonad-stimulating effect of implants of anterior hypophysis in hypophysectomized rats (Smith (6)) or normal immature rats (Evans and Simpson (7)) could be prevented by the simultaneous intraperitoneal injection of crude extracts of anterior hypophysis. Leonard (8) has shown similarly that simultaneous injection of Van Dyke's growth extract effectively masked the action of the follicle-stimulating fraction.

It may be noted here that the conditions employed for obtaining synergism are by subcutaneous injection of the *in vitro* combination of prolan with the synergic factor. The conditions used for demonstration of the antagonism phenomenon were subcutaneous injection of prolan and simultaneous intraperitoneal injection of the hypophyseal component.

In this investigation, the capacity of fractions containing the synergic factor to decrease the action of potent gonad-stimulators was determined and this property was almost invariably a concomitant of the synergistic activity (Table III). However, when digestion of anterior lobe was carried out (digestion with trypsin followed by a short digestion with erepsin) the two activities were differentially separated. The synergic factor was not injured but the digested material was no longer able to influence adversely the gonadotropic effect of prolan. Similarly an isoelectric precipitation at pH 4.4–4.6 of fractions exhibiting both phenomena left a supernatant liquor which was the most potent fraction in synergistic activity (Table VII) yet obtained, but was unable to decrease the effect of prolan. These

TABLE III

Antagonism and Synergism of Prolan and Anterior Hypophyseal Fractions with Subsequent Differential Destruction of the Antagonistic Factor

Preparation	Antagonism phenomenon					Synergism phenomenon					
	Hypophyseal fraction (intraperitoneal)		Prolan (subcutaneous)		Combination (for antagonism)	Hypophyseal fraction (subcutaneous)		Prolan (subcutaneous)		Combination (for synergism)	
	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Aqueous alcohol (pig); control, R3116	2.7	17	13.6	43	16	2.7	25	13.6	43	124	148
As above, pH 4.5, 70°C., 1 hr.					27					124	148
“ “ pH 4.5, 70° “ 3 hrs.					32					93	86
“ “ pH 8.5, 70° “ 1 hr.					27					79	68
“ “ pH 8.5, 70° “ 3 hrs.					35					54	8
Aqueous alcohol (sheep); control, R2289	2.7	25	13.6	43	24	1.36	21	13.6	38	137	234
As above, supernatant after isoelectric precipitation R3143	1.36	20			60	0.54		13.6	43	175	280
Trypsin and erepsin digest (sheep) R3356	1.36	19	13.6	45	46	1.36	25	13.6	31	144	278
	cc.										
Alkaline extract (sheep); control, R2889	1.5	9	13.6	30	20	2.7	63	13.6	35	178	122
						0.27	23			105	162
Above solution, trypsin digested	1.5	9			24	2.7	33			143	186
						0.27	20			64	73
Trypsin digestion followed by erepsin	1.5	12			47	2.7	37			153	183
						0.27	22			59	61

experiments (summarized in Table III) strongly indicate that the two properties are not due to the same constituent.

The fact that these fractions contain little or no growth hormone and do not contain the luteinizing or follicle-stimulating fractions would seem to eliminate the synergic material and these other substances from consideration as the antagonist.

TABLE IV

Combination of Synergic Factor with Prolan Prepared by Different Methods

Synergic factor			Prolan			Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Preparation	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*		*mg.*	*mg.*	*mg.*	*per cent*
Aqueous alcohol (sheep)	6.8	29	Alcohol-ammonia R2193	27.3	47	221	281
			Repeated alcohol precipitation R2194	13.6	31	111	164
				27.3	42	165	211
Aqueous alcohol (pig)	2.7	17	Alcohol-ammonia R2380	27.3	35	155	343
			Repeated alcohol precipitation R2381	54.5	33	136	312

Optimal Conditions for the Demonstration of Synergism

It seemed of interest to determine the effect of prolan variously prepared on the degree of synergism obtained. In this respect, prolan prepared by the alcohol-ammonia extraction method (9) was found to be much more potent than that prepared by repeated alcohol precipitation (see Table IV).

In order to determine the optimal proportions of prolan and the synergic factor, a constant amount of prolan was used in combination with varying amounts of the synergic principle and a constant amount of the synergic principle was used in combination with varying amounts of prolan. The results are given in Tables I and V. It is seen that for optimal augmentation between 10 and 20 times the minimal dose of prolan (gonadotropic assay) is necessary in combination with about two to three times the minimum dose of the synergic factor (activation assay). The use of larger doses of the synergic factor is inadvisable, since the augmentation produced is not as great

TABLE V

Combination of Variable Amounts of Prolan with Constant Amounts of Synergic Factor

Synergic factor			Prolan			Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Description of ovaries	Weight of ovaries	Activation
	mg.	*mg*	*mg.*	*mg.*		*mg.*	*per cent*
Aqueous alcohol (pig) R2739	1.36	22	0.54	26	Corpora, large follicles	33	10
			2.7	26	" "	108	260
			6.8	36	" "	114	185
			13.6	38	" "	130	210
			27.3	38	" "	187	345
			81.8	49	" "	209	294
	2.73	25	0.54	26	" "	44	33
			2.7	26	" "	91	176
			6.8	36	" "	154	258
			13.6	38	" "	131	191
			27.3	38	" "	158	251
			81.8	49	" "	105	87
Aqueous alcohol (pig) R3082	2.73	21	0.27	15	Infantile	26	24
			0.54	29	Corpora	37	16
			1.36	32	"	60	71
			2.73	30	"	75	127
			6.8	44	Corpora, large follicles	128	172
			13.6	43	" "	155	237

as that given by the lower doses. This may possibly be due to the presence of the antagonistic substance (*vide supra*) as a contaminant in the preparations then employed.

As to duration of the synergic experiments, Chart 2 indicates that a sufficient augmentation (with three injections) is obtained in 72 hours which is, therefore, not too early to terminate the experiment.

Chemical Characteristics of the Synergic Factor

The comparative stability of the synergic substance noted in the previous paper (1) has been shown to extend over a wide variety of hydrogen ion concentrations, even at slightly elevated temperatures. Thus in alkaline solution pH 8–12 it was stable at room temperature for 24 hours. At pH 8.5 it was seriously injured in 1–3 hours at 70°C. but at 37°C. it was stable for 6 hours. In acid solutions (pH 4–1) it was somewhat destroyed in 24 hours at room temperature. At pH 4–5 it was quite stable for 1–5 hours at 37°C., but 9 hours at pH 2 completely inactivated the material. Stability to acid and alkali is illustrated in Tables III and VI.

Enzymatic hydrolysis of anterior lobe material was next undertaken in order to remove as far as possible protein contaminants without destruction of the synergic principle. Pepsin was found to be unsatisfactory since the synergic factor was readily injured. Trypsin, however, was found to be quite advantageous since 4–5 hours digestion at 37°C. hydrolyzed a considerable portion of the protein, but did not affect the activity of the synergic principle. When the tryptic digestion was followed by erepsin, though some further hydrolysis was effected, there was little loss in activity. The erepsin digestion was of value since the antagonistic factor was thereby eliminated. The action of various digests is shown in Table VI.

Following tryptic and ereptic digestion, the solution could be concentrated *in vacuo* to one-tenth the original volume and then the addition of alcohol to a concentration of 80 per cent precipitated the active material which was potent for synergism in 0.27 mg. dose (3700 rat units per gm.). This material gave a positive biuret test.

The most convenient method of preparing a potent fraction of the synergic principle is the aqueous alcohol extraction procedure previously described (1). The material, however, contained appreciable amounts of the antagonistic factor among other possible contaminants.

Isoelectric precipitation of impurities was efficacious both in the elimination of the antagonistic factor and in producing the most potent fraction yet obtained. A potent fraction of the synergic factor (containing 7300 rat units per gm.) was precipitated isoelectrically and the supernatant liquor contained the active material which was precipitable

TABLE VI

Digestion of Sheep Anterior Pituitary Powder with Trypsin, Erepsin and Pepsin

Hypophyseal material			Prolan		Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	cc.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Alkaline extract, neutralized, frozen (control) R2798	2.7	63	13.6	32	178	131
	0.27	23			105	183
Alkaline extract (above), pH 8.5, 37°C. for 4.5 hrs. with trypsin R2803	2.7	33	13.6	32	143	204
	0.27	20			64	88
Trypsin digest (above), pH 8.0, 37°C. for 4 hrs. with erepsin R2807	2.7	37	13.6	32	153	200
	0.27	22			59	64
Alkaline extract, pH 4.0, 37°C. for 4.5 hrs. (control) R2811	2.7	23	13.6	32	139	276
	0.27	18			49	53
Alkaline extract, pH 4.0, 37°C. for 4.5 hrs. with pepsin R2813	2.7	18	13.6	32	46	43
	0.27	19			41	24
Aqueous alcohol extract water solution, neutral, frozen (control) R2711	0.27	32	13.6	38	140	169
Aqueous alcohol extract, water solution, pH 8.5, 37°C. for 6 hrs. (control) R2712	0.27	48	13.6	38	140	106
Aqueous alcohol extract, water solution, pH 8.5, 37°C. for 6 hours with trypsin R2713	0.27	28	13.6	38	196	308
Trypsin digest (above), pH 8.0, 3 hrs. with erepsin R2717	0 27	26	13.6	38	140	205

by alcohol. This material was potent in a total dose of 27 *gamma* (37,000 rat units per gm.). It still gave a biuret test. The biological assay of the active fractions described above (from digestion and from isoelectric precipitation) is shown in Table VII.

TABLE VII

Assay of Potent Fractions of the Synergistic Principle

Synergic principle			Prolan		Combination (*in vitro*)	
Preparation	Dose	Weight of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
Concentrate after tryptic and ereptic digestion (sheep) R3023	1.36	25	13.6	31	144	278
	0.54	—			134	252
	0.27	—			73	92
	0.136	—			46	21
Aqueous alcohol (sheep) R3372	0.27	28	13.6	45	125	127
	0.136	21			114	137
	0.54	19			57	24
Isoelectric supernatant from aqueous alcohol extract (sheep) R3500	0.011	32	13.6	34	143	198
	0.082	25			109	180
	0.054	21			113	205
	0.027	20			73	103

Biological Procedures

Unless otherwise specified experimental groups consisted of three female rats, 2–26 days of age at the beginning of the experiment. Daily injections were made n 3 consecutive days.

Standardization of gonadotropic substances was always made by subcutaneous jection. When testing for synergism the *in vitro* mixture of the two components as likewise injected subcutaneously. The desired amounts of prolan and synergic ctor were dissolved in a total of 11 cc. of water; each rat received a total of 3 cc. the mixture. Parallel tests were always made with each component of this xture; each substance was dissolved in 11 cc. of water so that the total volume liquid administered in control experiments and in the synergism experiments s the same.

n order to demonstrate antagonism, the hypophyseal fraction was always ad- istered intraperitoneally, and the prolan was simultaneously injected subcu- eously. Each component was dissolved in 5.5 cc. of water, with daily injection of 0.5 cc. of each solution per rat. In the control experiments each of the components used in combination was dissolved in 11 cc. of water and 1 cc. was injected daily. As in the combination experiment the prolan was injected subcutaneously and the hypophyseal component intraperitoneally.

All experimental animals were sacrificed 96 hours after the first injection unless otherwise specified. The ovaries were observed under a binocular microscope, then carefully dissected and weighed. The weights of ovaries given in the tables are averages based on three animals.

Preparation of Fractions Potent in the Synergic Substance

The aqueous alcohol extraction method previously described (1) has been found to be the most satisfactory method of preparing comparatively potent fractions of the synergic principle. Frozen sheep anterior pituitary glands (1370 gm.) were ground very fine into 5000 cc. of 60 per cent alcohol. The mixture was allowed to stand for 6 hours at room temperature with occasional stirring after which the solvent was decanted and filtered and the fluid saved (*ca.* 4500 cc.). The gland residue was reextracted with 5000 cc. of 40 per cent alcohol for 15 hours. The solvent was again decanted (*ca.* 4500 cc.) and the extraction repeated with 5000 cc. of 40 per cent alcohol for 3 hours. The combined filtrates (14,000 cc.) were poured into 32,000 cc. of alcohol and a small amount of saturated alcoholic sodium acetate was added to precipitate the active material. By this means there was obtained 6.0 gm. of a powder, almost completely soluble in water and potent in 0.136 mg. dose (R3372, Table VII).

Isoelectric precipitation of the above product eliminated inactive material and other impurities. For this procedure 1 gm. of the substance was dissolved in 100 cc. water and the insoluble material centrifuged off. The solution was adjusted to pH 4.4 and a large proportion of the material then precipitated. The mixture was allowed to stand 8 hours at room temperature and the precipitate was centrifuged off. Most of the activity was retained by the supernatant liquor which was poured into 500 cc. of alcohol and the active material precipitated by the addition of a small amount of saturated alcoholic sodium acetate. The yield was 0.16 gm. of a powder, potent in 0.027 mg. dose (R3500, Table VII) and giving a positive biuret test.

For the study of the action of enzymes on the anterior pituitary, with particular reference to the synergic and antagonistic principles, an alkaline extract of anterior lobe was used as stock material. Desiccated sheep anterior hypophysis (20 gm.) was extracted thoroughly with 1000 cc. of dilute sodium hydroxide solution (pH *ca.* 8–9) and the insoluble material centrifuged off (the yield of the insoluble fraction was 10.5 gm. and contained little of the synergic factor). The alkaline solution was adjusted to neutrality and used as the starting point for the following digestions. It contained about 1 per cent of the anterior lobe material.

The digestions with trypsin were carried out by adding 1 part of trypsin (Fairchild Bros. and Foster) to 100 parts of anterior lobe. The solutions were adjusted to pH 8.5 and kept at 37°C. throughout the digestion. The progress of the digestion was followed by determining the change in total acidity (10, 11). Aliquots of the solution were titrated at intervals with sodium hydroxide solution using thymolphthalein as the indicator. Digestion was found to be practically complete in 4 to 5 hours. Since no foreign buffer was used to maintain the hydrogen ion concentration, the solution was readjusted to pH 8.5 every 2 hours. The tryptic hydrolysis did not affect the synergic principle but digested much of the inactive protein present in the solution (Table VI).

Anterior lobe solution digested with trypsin was then subjected to digestion with erepsin. Erepsin (The Arlington Chemical Co.) was added in an amount equal to 1 per cent of the original concentration of anterior lobe, the solution adjusted to pH 8.0 and kept at 37°C. during the digestion. There was little change in titratable acidity during 4 hours but biological assay of the resulting solution indicated that the antagonistic factor had been destroyed with little harm to the synergistic principle (Tables III and VI).

Pepsin digestion of the alkaline extract of anterior lobe was carried out using 1 part of pepsin (The Wilson Laboratories) to 100 parts of anterior lobe material. Digestion at pH 4.0 and 37°C. completely destroyed the activity of the synergic principle within 4.5 hours (Table VI).

Solutions of the anterior hypophysis digested with trypsin and erepsin as described above gave no precipitate on addition of alcohol to 80 per cent concentration. The solutions were, therefore, first concentrated *in vacuo* (at a water bath temperature of 55°C.) to one-tenth the original volume. This concentrate on precipitation with four volumes of alcohol gave a product which was easily soluble in water and contained most of the activity present in the original solution. It was potent in 0.136 mg. dose and gave a positive biuret test.

SUMMARY

The hypophyseal substance—the synergic factor—which gives increased gonadotropic effects when combined with prolan has been shown to itself possess a definite though slight gonadotropic activity. It produces transitory follicular enlargement within 24–48 hours, which, however, regresses at once on cessation of treatment so that by the end of the 4th day the ovary again approximates its infantile weight.

The synergic principle indeed provokes only moderate ovarian development when administered in 100 times the dose necessary to demonstrate the activation phenomenon.

By means of isoelectric precipitation or by means of tryptic and ereptic digestion, fractions containing the synergic principle were freed of many contaminants, in particular of the antagonistic factor.

The synergic principle has been shown to be unaffected by digestion

with trypsin or trypsin followed by a short digestion with erepsin, but the action of pepsin inactivated the material.

A preparation of the active substance has been obtained which is potent in a total dose of 27 *gamma*.

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THE RECOGNITION AND COMPARISON OF PROLAN AND PROLAN-LIKE SUBSTANCES*

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It has been shown that the limited gonadotropic effect of prolan can be greatly enhanced by the simultaneous injection of a synergic principle from the hypophysis (1, 2). This suggested that the method could be used to demonstrate the presence of prolan or prolan-like substances occurring in blood or urines in conditions other than pregnancy.

The occurrence of a gonad-stimulating substance in the urine of men is manifested in certain pathological conditions (castration, genital carcinoma) (3, 4), but no evidence for the presence of such a substance in normal male urine has ever been obtained. In the course of this study, various methods for the concentration of prolan have been applied to male urine[1] and in the best concentrates (alcohol-ammonia extracts) it has been possible to demonstrate the presence of a prolan-like substance by administering it alone. Further, when these concentrates were strengthened by the addition of the synergic factor from the hypophysis, a significant augmentation of the infantile rat ovaries was elicited. This was the case with both the crude alcohol precipitate and the more concentrated extract (Table I).

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[1] We are grateful to Dr. J. A. Morrell of E. R. Squibb and Sons for generous supplies of a concentrate from urine of normal men.

It is a matter of no little concern to the physiologist that prolan has been found almost solely in the serum and urine of primates. With the sensitive synergism test at hand, it seemed important to inquire if small amounts of prolan-like substances could not be detected in the sera and urine of non-primates. Evidence to the effect that gonadotropic substances do in fact occur in mammals other than primates has accumulated from parabiotic studies (Matsuyama (5)). Martins (6) and Emery (7) found by direct injection that the blood of the castrate male and female rat contains a gonadotropic substance. The sera of normal males and pregnant or non-pregnant females gave only a negative reaction as did the urines of all animals studied. Jeffcoate (8) found that gonadectomized rabbits excrete a gonad-stimulating substance in their urine.

TABLE I

Response of the Immature Female Rat to a Combination of the Hypophyseal Synergic Principle with Prolan-Like Substances from Human Sources

Prolan-like substance				Synergic principle (pig)		Combination* (*in vitro*)	
Source	Total dose in 3 days	Weight of ovaries	Description of ovaries	Total dose in 3 days	Weight of ovaries	Weight of ovaries	Activation
	mg.	*mg.*		*mg.*	*mg.*	*mg.*	*per cent*
Pregnancy serum, acetone-ammonia extract R2438	54.4	36	2–6 corpora	2.7	25	82	90
	27.3	26	2–6 "			57	72
Placenta, alcohol precipitate of aqueous acetone extract R2384	54.4	20	Large follicles	2.7	20	94	327
	27.3	17	" "			38	90
Normal male urine (age 20–40 yrs.)							
Crude alcohol precipitate R2666	272	12	Infantile	2.7	32	66	106
Reprecipitated from alcohol R2346	163	15	"	2.7	17	54	200
Alcohol-ammonia extract R2662	272	16	3–8 corpora	2.7	32	81	165
Alcohol-ammonia extract R2442	215	28	3–4 "	2.7	25	53	61

* The given weights of the two components were mixed *in vitro* and injected subcutaneously, daily, on 3 successive days with autopsy after 96 hours (1, 2). Three rats were used in each group.

of rats led to a similar investigation of other animal forms. The findings in the rat were largely confirmed. Serum of pregnant and non-pregnant cow,[2] pregnant and non-pregnant pig and urines of pregnant and non-pregnant guinea pig and pregnant dog were all negative when injected alone and all gave activation when combined with the synergic factor. The urine of pregnant and non-pregnant rabbits,[3] however, showed but slight activation when tested similarly and the urine of pregnant mares, none at all. These results are summarized in Table III.

Since the activation phenomenon has been observed in many instances regardless of whether or not the prolan-like substances was positive by itself, it seemed worthwhile to give subminimal doses of prolan from pregnancy urine with the synergic factor. In Table IV is shown the effect of combining varying doses of prolan with a constant amount of the hypophyseal component. It was rather surprising to find that pregnancy prolan was potent in doses lower than those which showed the activation phenomenon. It is, therefore, apparent that the prolan-like material present in most non-pregnancy urine is different from that in pregnancy.

Because of the theoretical interest attached to the possible origin of prolan and prolan-like bodies from the hypophysis it seemed important to determine whether or not gonadotropic substances when actually injected into rats would reappear in their urine. Ehrhardt has shown (11) that the urine of a non-pregnant woman contained prolan shortly after she had received a blood transfusion from a pregnant woman.

In this study prolan and pregnant mare's serum were injected into separate groups of rats and their urine was collected, concentrated and assayed. Rats injected with prolan secreted at least a portion of it in their urine (quantitative recovery was not attempted). Both the native urine and the concentrates gave positive reactions. Thus 6 cc. of the sevenfold concentrated urine gave 62 mg. ovaries. However,

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[2] Leonard (9) has presented data that indicate the presence of prolan-like substances in the urine of pregnant and non-pregnant cows. Combination of the urinary product with the follicle stimulator (10) (which contains the synergic principle) gave increased ovary weights.

[3] We desire to thank Drs. Wade Brown and Louise Pearce for placing rabbits of known sexual history at our disposal.

TABLE III

Demonstration of a Prolan-Like Substance in the Urine and Blood Serum of Various Non-Primates

Prolan-like substance			Synergic factor		Combination (*in vitro*)		Prolan-like substance in male rats		
Source	Total dose in 3 days	Weight of ovaries	Total dose in 3 days	Weight of ovaries	Weight of ovaries	Activation	Total dose in 10 days	Weight of seminal vesicles	Weight of testes
		mg.	*mg.*	*mg.*	*mg.*	*per cent*		*mg.*	*mg.*
Pregnant cow blood serum R2668	3 cc.	16	2.7	22	78	254			
Normal cow blood serum R3459	3 cc.	13	1.36	18	73	305			
Pregnant cow blood serum, acetone precipitate R2632	272 mg.	15	2.7	22	57	159			
Pregnant pig blood, alcohol precipitate R3565	109 mg.	16	1.36	20	41	105	455 mg.	11	521
Normal female pig blood, alcohol precipitate R3566	109 mg.	18	1.36	20	68	240	10 cc. (serum)	10	457
(Controls)							10 cc. (serum)	7	563
								(9)	(446)
Pregnant guinea pig urine, alcohol precipitate R3491	Equivalent of 41 cc. urine	16	1.36	18	64	255			
Normal female guinea pig urine, alcohol precipitate R3493	Equivalent of 30 cc. urine	13	1.36	18	31	61			
Pregnant dog urine, alcohol precipitate R3495	Equivalent of 35 cc. urine	14	1.36	22	34	54			
Pregnant rabbit urine, alcohol precipitate R3477	Equivalent of 75 cc. urine	17	1.36	22	39	77			
Normal female rabbit urine, alcohol precipitate R3473	Equivalent of 80 cc. urine	17	1.36	22	27	0			

of rats led to a similar investigation of other animal forms. The findings in the rat were largely confirmed. Serum of pregnant and non-pregnant cow,[2] pregnant and non-pregnant pig and urines of pregnant and non-pregnant guinea pig and pregnant dog were all negative when injected alone and all gave activation when combined with the synergic factor. The urine of pregnant and non-pregnant rabbits,[3] however, showed but slight activation when tested similarly and the urine of pregnant mares, none at all. These results are summarized in Table III.

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urine from rats receiving pregnant mare's serum could not be differentiated from urine of uninjected controls. This is in agreement with the fact that no gonadotropic hormone is found in the urine of the pregnant mare.

Realizing that the physiological mechanism of the rat differs from that of primates in the handling of gonadotropic substances, we injected *rhesus* monkeys intravenously with pregnant mare's serum and hypophyseal gonad-stimulating substance (pig flavianate preparation). The urines were collected, concentrated and assayed in immature female rats. A gonadotropic hormone was abundantly demonstrated in the urine of the monkeys injected with the hypophyseal hormone. An ovary weight of 62 mg. was elicited by the injection of a total dose of 69 mg. of the urinary concentrate. Combination with the synergic substance did not further increase the ovary weights. The urinary product from the monkeys injected with pregnant mare's serum contained no gonadotropic substance. The results were, therefore, comparable to those in the rat. Further, the urine did not show the activation phenomenon on combination with the synergic principle. The urine from normal controls did not cause ovarian development when injected alone or in combination with the synergic factor.

TABLE IV

Effect of Combination of Varying Doses of Prolan with a Constant Amount of the Synergic Principle from the Hypophysis

Prolan (R3082)			Synergic principle (pig)		Combination (*in vitro*)	
Dose	Weight of ovaries	Description of ovaries	Dose	Weight of ovaries	Weight of ovaries	Activation
mg.	*mg.*		*mg.*	*mg.*	*mg.*	*per cent*
13.6	43	Large follicles and corpora	1.36	21 (Small and medium follicles only)	155	237
6.8	44	" "			128	172
2.7	30	" "			75	127
1.36	32	" "			60	71
0.54	29	Few corpora			37	15
0.27	15	Infantile			26	23

Since the synergic principle from the hypophysis has such a pronounced effect on the ovaries when combined with prolan it was of interest to see if there was a similar effect on the seminal vesicles.

TABLE V

Response of the Immature Male Rat to a Combination of the Synergic Principle from the Hypophysis with Prolan from Pregnancy Urine

Material injected	Injected 3 days, sacrificed 5th day, R1989			Injected 11 days, sacrificed 12th day, R2276			Injected 19 days, sacrificed 20th day, R2658		
	Dose	Weight of seminal vesicles	Weight of testes	Dose	Weight of seminal vesicles	Weight of testes	Dose	Weight of seminal vesicles	Weight of testes
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Prolan	27.3	33	471	110	85	610	180	125	900
Synergic principle	27.3	7	267	11	7	395	18	26	805
Prolan plus synergic principle		49	535		119	749		372	1362
(Controls)		(8)	(308)		(6)	(498)		(13)	(483)

TABLE VI

Response of the Immature Male Rat to a Combination of the Synergic Principle from the Hypophysis with Prolan-Like Substances from Human Sources

Prolan-like substance				Synergic principle		Combination (*in vitro*)			Controls	
Source	Total dose in 10 days	Weight of seminal vesicles	Weight of testes	Total dose in 10 days	Weight of seminal vesicles	Weight of testes	Weight of seminal vesicles	Weight of testes	Weight of seminal vesicles	Weight of testes
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Pregnancy urine R2276	110	85	610	11	7	397	119	749	6	492
Pregnancy serum R3066	90	54	689	4.5	15	730	41	785	9	444
Normal male urine R2277	660	6	365	11	7	397	7	613	6	492

This was found to be the case: The synergic principle itself had little or no effect, but in combination with pregnancy prolan it caused a definite increase in the development of the seminal vesicles (Table V). These results also indicate that the 10 day test is adequate for the attainment of significant values.

The action of prolan-like substances from various sources on the testes and seminal vesicles is shown in Table VI and also in Tables II and III. It is perhaps significant that all of the non-primate sources, shown to contain a prolan-like substance as evidenced by the activation phenomenon with immature females, were uniform in their lack of effect on the testes and seminal vesicles. Normal male urine conformed to the above classification.

SUMMARY

The synergism phenomenon has made possible the recognition of substances, which we have called "prolan-like," in a wide variety of conditions. Indeed, it has been possible to demonstrate a prolan-like material in the urine of normal men.

The method has shown a wide distribution of prolan-like substances in sera and urines of non-primates without demonstrating, however, significant difference in the reactions secured from pregnant as contrasted with non-pregnant states, or in males as contrasted with females.

The synergism phenomenon with pregnancy prolan can also be shown in the increased development of the seminal vesicles of immature male rats. However, such males cannot be advantageously employed in the detection of prolan-like gonadotropic substances occurring in the blood and urine in conditions other than pregnancy, for the prolan-like substances usually do not effect appreciable development of the seminal vesicles.

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CONCENTRATION OF THE GONADOTROPIC HORMONE IN PREGNANT MARE'S SERUM*

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In 1930, Cole and Hart (1) discovered the presence of a gonadotropic hormone in the blood stream of mares in early pregnancy. It was detected almost simultaneously by Zondek (2). Cole and Hart established the following facts. The hormone is present in the period beginning at 37 days after the fertilizing coitus and lasts until approximately the 175th day but the presence of very appreciable amounts of it is limited to the period between 43 and 80 days. The period of greatest concentration occurs apparently somewhere in the interval between the 50th and 65th day and the native unaltered serum drawn from the mare's jugular vein at this time is capable of exerting clear gonadotropic effects in immature (21–26 day old) female rats or mice within a hundred hours following six doses of 0.005 cc. These investigators and others associated with them have continued study of the very interesting hormonal and tissue changes in pregnant mares and in the horse fetus (3–7). They have shown that during the period of high concentration of gonadotropic hormone in the mare's blood stream, its ovaries exhibit the formation *de novo* of many corpora lutea, an unique phenomenon; and that in the period immediately subsequent thereto, the fetal gonads exhibit an astonishing hypertrophy caused by the massive appearance there of interstitial cells.

It is apparent that equine hormonal and tissue conditions are fully as remarkable as those which characterize primates (with the Aschheim-Zondek reaction of pregnancy blood and urine) and that further inquiry here will be well repaid. Evans, Meyer and Simpson (8) included the hormone from the mare's blood in their comparative study of gonad-stimulating substances from the hypophysis and other

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sources and showed the relatively great concentration of gonadotropic hormone in this source.

The chemical properties of the gonadotropic substance of pregnant mare's blood have been studied by Goss and Cole (5) and by Cole, Guilbert and Goss (6). They found it possible to concentrate the serum at 36°C. in partial vacuum without appreciable loss of potency. Indeed, heating the serum to its coagulation point and even to the temperature of the boiling water bath for several minutes failed to destroy the gonadotropic principle. The active material failed to dialyze through a collodion membrane and could not be detected in ultrafiltrates. No effect was produced when massive doses of serum were fed to rats.

In agreement with Cole, Guilbert and Goss (6) we have found the active principle to be much more stable to alkalies than to acids at ordinary temperatures. No loss of potency could be observed in samples which had been allowed to stand 15 hours at 22°C. in range pH 3.7 to pH 11.0. Below pH 3.7 the loss of potency was distinctly apparent and potency decreased with increasing acidity.

Pepsin had little effect on the gonadotropic substance when the digestions were carried out at pH 4–5 at 37°C. for 4 hours. At pH 1.8–2.0 the activity was destroyed but this may not have been due to the action of pepsin since controls in which no pepsin was used were also inactivated at this pH and temperature.[1] Trypsin at pH 8.5 inactivated the hormone after 4 hours at 37°C. The products of peptic and tryptic digestion did not increase the gonadotropic effect of pregnancy prolan.[2] Inactivation was also observed when solutions at pH 7.5 were heated for 4 hours at 60–80°C. and the resulting product showed no tendency to increase the gonadotropic effect of pregnancy prolan.

When the acetone-dried serum was dissolved in anhydrous formic acid for 2 hours at 25°C. and the serum proteins recovered by precipitation with acetone, it was found that the gonadotropic activity had been lost and no increase in the activity of pregnancy prolan was observed when the inactivated material was injected with it.

The crude serum of the pregnant mare shows a very considerable

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[1] Cole, Guilbert and Goss (6) found partial inactivation of the hormone followed peptic digestion at pH 3.

[2] *Cf.* Evans, H. M., Simpson, M. E., and Austin, P. R., *J. Exp. Med.*, 1933, 57, 897.

gonadotropic potency. Cole and Hart (1) found the maximum ovarian response in rats followed 6 injections of 0.2 cc. of serum from a mare 78 days pregnant, autopsy being performed 96 hours after the first injection. Increasing the dose to 0.5 cc. showed no further increase in effect, while as has been mentioned, as little as 6 injections of 0.005 cc. of crude serum produced ovarian effects which could be detected by histological section.

Goss and Cole (5) attempted to concentrate the active principle by fractional precipitation of the serum with salts. They found that the fraction precipitable between the concentrations 20 and 27 per cent sodium sulfate contained the greater part of the activity while only 11 per cent of the serum protein was found in this fraction. Evans, Meyer and Simpson (8) prepared acetone-ammonia extracts from the acetone-dried serum and precipitated solutions of these acetone-ammonia extracts with flavianic acid. These procedures gave potent materials still contaminated by inactive serum protein.

With the hope of finding a better method of attack on the problem of the isolation of the active agent we have made a comprehensive study of the behavior of the hormone towards a number of adsorbents following the technique which has yielded conspicuous success in the purification of enzymes by Willstätter and his school. This study has shown that aluminum hydroxide preparations are excellent adsorbents for the gonadotropic substance found in the serum of the pregnant mare.

When aqueous solutions of the acetone-dried proteins of pregnant mare's serum were adjusted to pH 3.5 and treated with suspensions of aluminum hydroxide (Willstätter Type A and Type B) (9) it was found that the active substance was readily adsorbed on the surface of the aluminum hydroxide. In this way it has been possible to separate the active agent from a large part of the inactive protein material. Subsequent washing of the aluminum hydroxide on which the hormone had been adsorbed, using acetate buffer at pH 3.5 or water, removed practically none of the active material. The elution of the active substance was, however, easily accomplished by dilute ammonia or by converting the aluminum hydroxide into the basic phosphate with disodium phosphate solution in the usual manner. The active solutions

0.012 mg.) At the end of 10 days the testes weighed 1516 mg. and the seminal vesicles 200 mg. (the control organs weighed respectively 495 mg. and 8 mg.). After 30 daily injections the testes weighed 2620 mg. and the seminal vesicles weighed 979 mg. (control organs weighed 1155 mg. and 13 mg. respectively). Histologically it was found that after 10 daily injections the testicular tubules were already noticeably increased in diameter and that there was a marked increase in interstitial tissue. After 30 days the tubules were even larger, the spaces between tubules were densely packed with interstitial tissue and spermatozoa were found in the lumen of the tubules.

SUMMARY

The gonadotropic hormone of the blood of the pregnant mare has been greatly concentrated by adsorption on active aluminum hydroxide followed by elution. The preparations so obtained gave demonstrable gonadotropic effects within 100 hours in 21 day old female mice following three subcutaneous injections of 0.001 mg. in 1 cc. of physiological saline.

As is well known, other gonadotropic substances do not cause conspicuous development of the male gonads but injections of comparatively large doses of these preparations into immature male rats caused marked development of the testes, which in 10 days were trebled in weight. An astonishing increase in the weight of the seminal vesicles resulted, for these organs were approximately 75 times heavier than in controls.

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THE CULTIVATION OF MONOCYTES IN FLUID MEDIUM

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Plate 29

(Received for publication July 13, 1933)

The purpose of the experiments described in this article was to ascertain the conditions under which monocytes could be cultivated in a fluid medium. The plasma coagulum that is customarily used in the Carrel technique[1] for the cultivation of these cells seriously interferes with quantitative measurements of their metabolism. For such studies, it would be of great advantage to replace it with a liquid. That monocytes can live for a time in fluid has been shown by the work of Lewis and Lewis,[2] and also that of de Haan.[3] In the experiments of Lewis and Lewis,[2] the monocytes lived in hanging drops of liquefied plasma for 2 to 4 weeks, feeding partly, at least, on the other cells of the blood. In de Haan's experiments,[3] they were cultivated for 2 to 3 weeks in a slowly flowing stream of peritoneal exudate. De Haan[3] has already made some measurements of their metabolism under these conditions. The fact that his technique requires very large volumes of fluid limits its usefulness to a certain extent. Moreover, peritoneal exudate does not contain all the ingredients necessary for the prolonged cultivation of monocytes. De Haan[3] supplemented its nutritive properties by the addition of polymorphonuclear leucocytes from time to time. In the experiments described in this article, an effort was made to find a means whereby monocytes could be cultivated

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[1] Carrel, A., *J. Exp Med.*, 1923, 38, 407. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, 36, 365; 1926, 44, 285.

[2] Lewis, M. R., *Am. J. Path.*, 1925, 1, 91. Lewis, M. R., and Lewis, W. H., *J. Am. Med. Assn.*, 1922, 84, 798. Lewis, W. H., *Harvey Lectures*, 1925–26, 21, 77. Lewis, W. H., *Arch. exp. Zellforsch.*, 1928, 6, 253.

[3] de Haan, J., *Bull. histol.*, 1927, 4, 293; *Arch. exp. Zellforsch*, 1928, 6, 276; 1930-31, 10, 82. de Haan, J., Kolk, K. H., and Gerritsma, H., *Arch. exp. Zellforsch.*, 1928, 7, 283; 1929, 8, 452.

for a longer time in a small volume of fluid without using other cells as food.

Plan of the Experiments

The experiments of Carrel and Ebeling[1] have indicated that monocytes cultivated in coagulated plasma feed upon the serum rather than the fibrin. Yet, up to the present, all attempts to grow them in serum without a fibrin substratum have failed. It seemed probable that this was due to the fact that in a coagulum acid, and perhaps other metabolites, accumulate around the cell colony, thus creating the conditions necessary for its survival, while in the absence of a coagulum, the products of metabolism are quickly removed from the vicinity of the cells.

As early as 1913, Rous[4] showed by adding litmus to the medium that sarcoma and embryonic tissue embedded in coagulated plasma gave rise to enough acid within a few hours to turn blue litmus red in the immediate vicinity of the tissue. This has since been found to be true of monocytes. When measured with the glass electrode of MacInnes and Dole,[5] it was found that the pH of the medium immediately surrounding a piece of chick embryo spleen dropped in half an hour from 8.0 to 7.2. In 24 hours, it fell to 6.8, and in several days to 6.2.[6] Aside from this acid production, very little is definitely known about the changes in the medium surrounding a colony of monocytes.

The problem was approached, therefore, by ascertaining first the pH at which chicken monocytes would survive longest in serum. For this, homologous serum at 25 per cent concentration in Tyrode solution was used and adjusted by means of lactic acid to pH values ranging from 6.0 to 7.8. Then, a comparison was made of the relative value of using lactic acid, hydrochloric acid, and carbon dioxide for regulating the acidity. Finally, serum at the optimum pH value was modified by the addition of a variety of substances in an attempt to find a medium in which the monocytes would live, and to reproduce, if possible, the conditions that exist around a colony of

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[4] Rous, P., *J. Exp. Med.*, 1913, **18**, 183.

[5] MacInnes, D., and Dole, M., *J. Gen. Physiol.*, 1929, **12**, 805.

[6] Baker, L. E., unpublished experiments.

monocytes embedded in plasma. Glutathione was used because of its reducing action, and its rôle in cell respiration.[7] Proteins hydrolyzed to varying degrees by enzymes were tried, because it is known that monocytes will digest particles of protein which they have ingested,[8] and also because hydrolyzed proteins have been found to increase enormously the proliferation rate of monocytes[9] when incorporated in their medium. Particles of denatured protein, coagulated egg white, and dead muscle, which monocytes feed upon,[8] were also used. Various combinations of nucleic acid, hemoglobin, glutathione, and amino acids or digested proteins were tried, since they have been found beneficial in the building up of a synthetic medium for fibroblasts.[10]

Technique

For these experiments, the following technique was used. A tiny fragment of chick embryo spleen or of blood leucocytic film was fastened to the bottom of a Carrel D-3 flask by means of a drop of 25 per cent plasma. The plasma was spread over about 2 sq. cm. of the surface of the flask, and coagulated with a trace of tissue juice. 1.5 cc. of experimental fluid was then added. This procedure allowed the cells to form a colony instead of scattering throughout the whole medium, and made it possible to measure their rate of migration for several days. At the same time, this small coagulum was so very thin that the composition of the medium within it could not differ appreciably from that of the fluid as a whole. After a few days, the original fragment was extirpated from the clot. Later, when quite a number of cells had migrated onto the glass, or had been deposited there from the fluid, the tiny clot and the cells it contained were also removed. In this way, any possible effects of the original tissue and of the coagulum were eliminated. From this time on, the entire medium was liquid.

The changes in hydrogen ion concentration of the medium were followed in each culture day by day. In some experiments, this was done by removing a drop of the medium and testing it on a drop plate with indicators. In other experiments, phenol red at a concentration of 0.005 per cent was incorporated in the medium, and comparison made with standard flasks containing the same amount of phenol red in buffers at known pH values.

Adjustment of the pH of the medium with lactic or hydrochloric acids was made just before adding the fluid to the culture. With CO_2, it was accomplished by

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[7] Hopkins, F. G., *Biochem. J.*, London, 1925, 19, 787.

[8] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, 44, 285.

[9] Baker, L. E., *J. Exp. Med.*, 1933, 57, 689.

[10] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, 47, 353. Baker, L. E., *J. Exp. Med.*, 1929, 49, 163.

replacing the air of the culture flask with a gas mixture containing 21 per cent oxygen, sufficient carbon dioxide to give the desired pH value, and nitrogen. This method has been used now for some time to adjust the pH of cultures made with coagulated plasma. With the fluid cultures, the gas was introduced immediately, and contained a higher percentage of carbon dioxide in order to lower the pH value to a greater degree. The concentration of carbon dioxide necessary was determined, of course, by the concentration of serum and other buffering substances in the medium, as well as by the pH desired.

EXPERIMENTAL RESULTS

Effect of the Hydrogen Ion Concentration.—It was found that the monocytes proliferated most rapidly when the initial pH of the medium was 7.2 to 7.4. As long as the pH was not allowed to fall below 7.0 or 6.8, the cells remained clear, active, free from dark granulations, and showed no tendency to degenerate (Fig. 1). They remained in excellent condition, even after the original tissue and the small coagulum had been removed from the flask.

Effect of Different Acids.—Equally good results were obtained with all three acids (lactic, hydrochloric, and carbon dioxide), both in respect to the rate of proliferation and the condition of the cells. Adjustment with carbon dioxide was adopted as the routine procedure, however, since it does not destroy the buffer action of the medium, as the other acids do, and is also more convenient to use.

Effect of the Concentration of Serum.—Increasing the concentration of serum in the medium caused an increase in the rate of proliferation of the monocytes. In some sera, this continued up to 100 per cent concentration. Other sera, however, appeared to be toxic at concentrations above 50 per cent. In 25 per cent serum, multiplication was slow, but the cells remained active and relatively clear, with small cytoplasmic structures. In 50 per cent serum, the monocytes proliferated more rapidly. They also became larger and contained more and larger granules and fat globules. In very high concentrations of serum, they ultimately deteriorated.

Effect of Added Metabolites or Nutrients.—None of the metabolites or nutritive materials added to the serum improved its value as a culture medium for the monocytes. Glutathione, nucleic acid, hemoglobin, and sodium lactate had no beneficial action at the concentrations tried in these experiments. Particles of egg yolk, denatured albumin, and heated muscle were ingested by the cells, as is usual in a coagulum,[6] but the cells became large, round, dark, and granular, and appeared inert. The products of tryptic digestion of protein increased the rate of proliferation of the monocytes, as they do in plasma,[9] but were not necessary to their life. The cells became more fatty, and larger numbers of them died when the digestion products were added to the medium. On the other hand, serum alone appeared to be entirely adequate for the nutrition of the monocytes.

Description of Colonies Cultivated for 2 Months in Serum

Several colonies of monocytes, both from blood and from spleen, have been cultivated now for over 2 months in 25 per cent and also in 50 per cent serum (Fig. 2). They are still as active as at the beginning of the experiment and give every evidence from their appearance that life and proliferation will continue indefinitely, if the same conditions of cultivation are maintained. Throughout this time, the medium was adjusted to an initial pH of 7.4. A gas mixture containing 3 per cent CO_2 was used with 25 per cent serum, and one containing 5 per cent CO_2 with 50 per cent serum. New medium was given when the pH had fallen to 7.0 or 6.8. In those colonies in which the pH was allowed to fall below this value without renewing the medium, the cells became larger and more granular (Fig. 3). They also accumulated large refractile globules and showed a tendency to adhere to each other. They did not die even when the pH fell to 6.4 or somewhat below. At first, when only a small number of cells was present, renewal of the medium once in 4 days was sufficient. Later, new serum was supplied every 24 hours. After a time, so many cells accumulated in the flask that a portion of them had to be removed to prevent the pH from falling below the desired value.

Most of the cells adhered firmly to the glass surface of the flask. Those that did come loose were deposited on the glass at a distance from the colony and started a new colony. The lymphocytes and polymorphonuclear leucocytes that were present at the beginning soon disappeared, as they do in coagulated plasma. The fibroblasts from the spleen grew out less rapidly than the wandering cells and were replacing the air of the culture flask with a gas mixture containing 21 per cent oxygen, sufficient carbon dioxide to give the desired pH value, and nitrogen. This method has been used now for some time to adjust the pH of cultures made with coagulated plasma. With the fluid cultures, the gas was introduced immediately, and contained a higher percentage of carbon dioxide in order to lower the pH value to a greater degree. The concentration of carbon dioxide necessary was determined, of course, by the concentration of serum and other buffering substances in the medium, as well as by the pH desired.

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Most of the cells adhered firmly to the glass surface of the flask. Those that did come loose were deposited on the glass at a distance from the colony and started a new colony. The lymphocytes and polymorphonuclear leucocytes that were present at the beginning soon disappeared, as they do in coagulated plasma. The fibroblasts from the spleen grew out less rapidly than the wandering cells and were removed when the original fragment and tiny coagulum were extirpated. Therefore, a pure strain of monocytes was obtained. Instead of scattering and migrating away from each other, as they do in coagulated plasma, the monocytes, both in the tiny clot and on the glass, remained in close proximity. As multiplication proceeded, they spread out in a monocellular layer, covering the entire surface of the flask and almost touching each other. The appearance (Figs. 1 and 2) of the individual cells was entirely like that already described by Lewis and Lewis[2] in their studies of monocytes adhering to the cover-slip in hanging drop cultures. They flattened out on the glass in a more or less circular form (Figs. 1 and 2), presenting a very different appearance than they do as they migrate through a fibrin coagulum (Fig. 4).

The small monocytes were gradually transformed into macrophages, as always occurs in cultures of these cells when they are well fed. Some giant cells were formed, as has also been reported by Lewis and Lewis,[2] and de Haan.[3] Smaller monocytes adhered to these giant cells and appeared to be sucked into them, or to unite with them, just as Lewis[2] has described. In these experiments, however, the number of giant cells was very small in comparison with the number of normal macrophages.

The cells continued to proliferate throughout the entire period of cultivation. In less than a month, the whole surface of the flask was covered with them. To test their continued ability to proliferate, the cells on one-half of the surface of the flask were scraped off with a platinum spatula. These, together with the medium, were transferred to another flask. New medium was then added to both flasks, and cultivation continued. The cells that had been suspended in the fluid soon adhered to the surface of the new flask and continued to proliferate. In 2 to 3 weeks, the entire surface of this flask was covered with cells. The monocytes in the original flask also increased in number so that the half of the flask that had been freed from cells was again covered with them in a short time. In order to continue their cultivation, large areas of cells were removed from the flask every few days. Otherwise, too great a decrease in the pH value of the medium or exhaustion of its food constituents would have occurred.

Subcultivation of Cells Suspended in the Fluid

Though the great majority of monocytes adhered firmly to the glass, a few of them were usually to be found in the fluid when it was removed. That these cells were alive and in good condition was shown by cultivating them again in a coagulum. The fluid was centrifuged and the few cells it contained were taken up in a drop of plasma containing some embryonic juice. This was transferred to a new flask and allowed to coagulate. Enough dilute plasma was then added to cover the rest of the surface of the flask, and the pH was brought down immediately to 7.2 with carbon dioxide. After a few days, the cells migrated into the outer coagulum. Fig. 4 shows cells cultivated in this way after 12 days' sojourn in fluid medium.

DISCUSSION

In the experiments described in this paper, it has been shown that monocytes can be cultivated in pure strain in a fluid medium. Serum diluted with Tyrode solution supplied all the necessary food material. At the end of 2 months' cultivation, the cells were still in excellent condition, multiplying rapidly, and giving every indication that they would continue to proliferate indefinitely under the same conditions. Cultivation in dilute serum without a coagulum was made possible by immediately reducing the pH of the medium to 7.4 or 7.2, and not allowing it to fall below 7.0 or 6.8 during incubation. Monocytes embedded in a coagulum at a higher pH value survive only because their combined metabolism lowers the pH around them to this value or a lower one. The cells die in a fluid in which it is not possible for them to lower the pH to a sufficient extent. It seems probable that the monocytes cultivated in the hanging drop cultures of Lewis and Lewis[2] lived because the conditions of those experiments either did not permit so much loss of CO_2 from the medium as takes place in the Carrel flasks, or because the number of cells present was great enough to reduce the pH of the single drop of medium to a value that permitted their survival. De Haan[3] does not lay very great stress on the hydrogen ion concentration required for cultivation of monocytes in his flowing medium. In some experiments, he saturated the fluid with 7 per cent CO_2. In others, the original pH value of the medium was 8.0.

He states, however, that the pH in the chambers containing the cells varied from 7.2 to 6.8. This agrees well with the present experiments. Even if the original pH was 8.0, a large number of cells in a small chamber could reduce it to 7.2, especially if it were not well buffered. Cultivation in Carrel flasks at higher pH values than those used in these experiments might be successful, if enough cells were present to lower the pH quickly, or if the medium had a smaller buffer value. In this case, new serum would have to be supplied more often.

In the first days of cultivation, the monocytes undoubtedly fed to some extent on the other cells present. However, cultivation was continued so long after the lymphocytes and polymorphonuclear leucocytes disappeared that it was evident that these other cells are not needed for food if fresh serum is supplied fairly often. Moreover, monocytes transferred to a new flask, after a pure strain had been obtained, continued to proliferate in serum without any other food material.

No attempt has been made as yet to cultivate isolated or even a very small number of cells under these conditions. It is quite possible that single cells would require still different conditions from those described here.

SUMMARY

Monocytes from blood and from spleen have been cultivated in fluid medium in Carrel flasks for over 2 months. Diluted serum supplied all the essential nutritive substances. Cultivation in fluid was made possible by adjusting the initial pH of the fluid to 7.4, and not allowing it to fall below 7.0 or 6.8. The cells remained in good condition when the pH was adjusted with either lactic acid, hydrochloric acid, or carbon dioxide. Adjustment with carbon dioxide was found to be more convenient and also more practical, since it does not destroy the buffer action of the medium. After 2 months of cultivation, the monocytes were in excellent condition and still proliferated actively. They gave every indication that indefinite multiplication could be maintained under the conditions of these experiments. It is hoped that this method of cultivation, with some modifications, will prove useful in studying the metabolism of these cells.

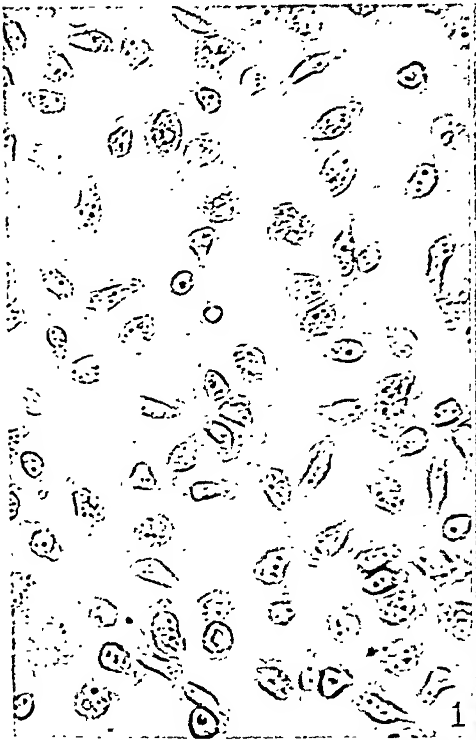
EXPLANATION OF PLATE 29

Fig. 1. Monocytes from chick embryo spleen cultivated in 25 per cent serum for 30 days. The medium was adjusted initially to pH 7.4 with carbon dioxide, and not allowed to fall below 7.0 during cultivation. ×230.

Fig. 2. Monocytes from chick embryo spleen cultivated in 50 per cent serum for 64 days. The pH fell below 6.8 several times during cultivation. ×230.

Fig. 3. Monocytes from chick embryo spleen cultivated in 50 per cent serum for 2 months. So many cells were present that the pH fell repeatedly below 6.8 during 24 hours' incubation. Consequently, the cells are loaded with granulations and show a tendency to agglutinate. × 230.

Fig. 4. Monocytes obtained from the fluid of a culture, which had been 12 days in liquid medium, now being cultivated in a coagulum. ×230.



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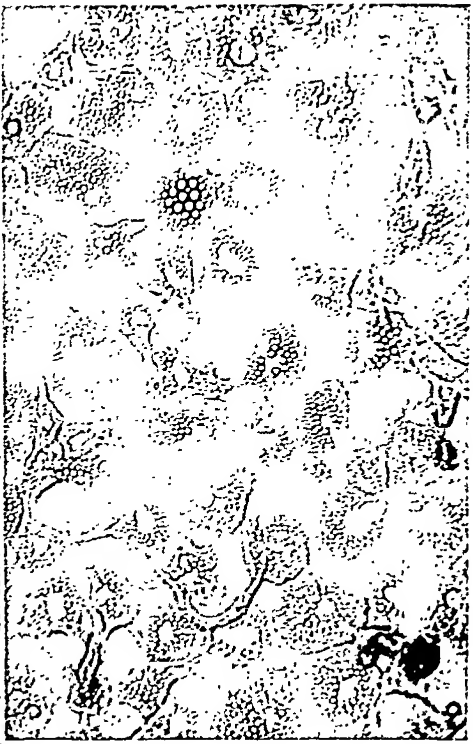

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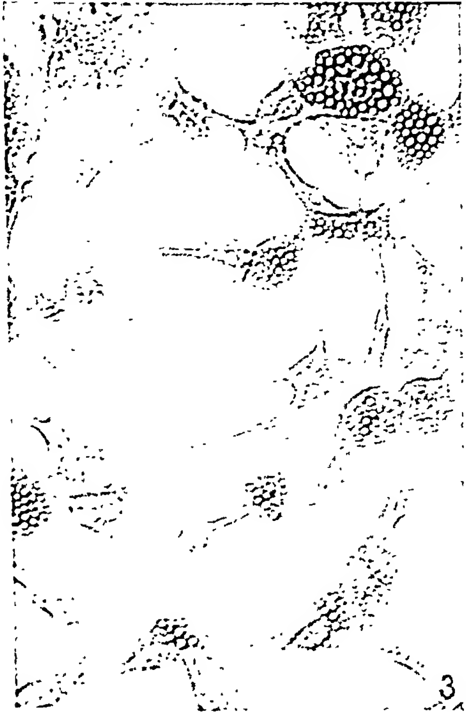

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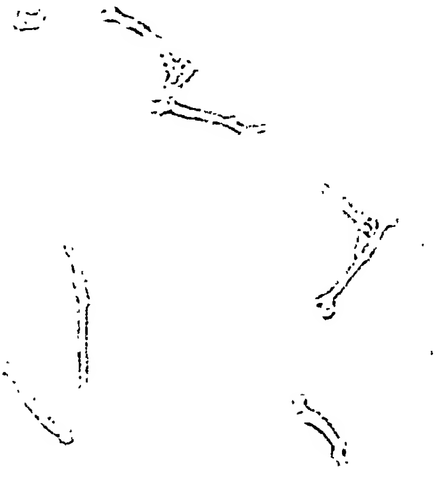

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THE PRODUCTION IN DOGS OF CHRONIC BLACK TONGUE WITH ANEMIA

By C. P. RHOADS, M.D., and D. K. MILLER, M.D.

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Plates 30 to 32

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Glossitis, stomatitis, and gastrointestinal disturbances are outstanding features of three diseases of human beings: pellagra, sprue, and pernicious anemia. The same triad of symptoms characterizes an acute, spontaneous or experimental disease of dogs, known as black tongue. Experimental evidence indicates that pellagra in human beings and black tongue in dogs can be produced by diets lacking in a particular accessory food factor, and can be successfully treated by supplying that factor. These facts established the symptomatic similarity between the two conditions on the basis of a like etiology. The demonstration by Castle and Rhoads[1] that the same accessory food factor, vitamin B_2 or G, is effective therapeutically in sprue, and by Strauss and Castle[2] that it forms a part of a complex therapeutic agent capable of effecting remissions in pernicious anemia, has suggested that lack of that factor may play a rôle in the etiology of both conditions. Proof of this hypothesis required the production in animals of a disease condition more like sprue and pernicious anemia than was acute black tongue of dogs. If in addition to the oral and intestinal symptoms which are so striking in that disease the features of chronicity and anemia of a characteristic type were added, an experimental syndrome would be established symptomatically like sprue and pernicious anemia in man. Accordingly the production of chronic recurrent black tongue in dogs, with a study of the associated alterations of the blood, was undertaken. The results obtained are presented in this communication.

[1] Castle, W. B., and Rhoads, C. P., *Lancet*, 1932, **1**, 1198.

[2] Strauss, M. B., and Castle, W. B., *Lancet*, 1932, **2**, 111.

A rather comprehensive literature exists concerning black tongue in dogs. In 1907 Chittenden[3] observed that dogs deprived of meat and milk fell ill with the salivation, stomatitis, and diarrhea now recognized as being so characteristic of that condition. The disease picture was similar to one called Stuttgart disease which had been described as occurring spontaneously in Europe. In the southern states of North America it was known as black tongue. In 1917, with Underhill, Chittenden[4] produced a pathological condition marked by salivation, stomatitis, and diarrhea, by feeding dogs a diet composed of boiled peas, cracker meal, and cottonseed oil. It was considered to be similar in all respects to spontaneous black tongue.

The most numerous and detailed experiments dealing with this condition are those of Goldberger and his coworkers.[5-10] These experiments may be epitomized as follows: By the use of a diet composed principally of white corn-meal plus dry peas, casein, cod liver oil, cottonseed oil, and a salt mixture, an acute illness of dogs could be produced in a period of from 4 to 10 weeks. This illness was of brief duration and was marked by stomatitis, salivation, diarrhea, and a fatal outcome. It was very much like the disease produced by previous workers, and was supposed to be experimentally produced black tongue. Untreated animals rarely survived more than a few days. Dermatitis and elevation of temperature were frequently observed. Denton[11] studied the histopathological changes in the tissues of the dogs dying of black tongue in the course of the experiments carried out by Goldberger. The findings were compared with those in human pellagra and were considered to be very similar. From the symptomatic and pathological resemblance, black tongue was considered to be pellagra in dogs. Accordingly a long and detailed series of experiments was performed to ascertain whether the experimental condition in the dog could be prevented and relieved by those foods which appeared to be of prophylactic and therapeutic value in pellagra. A remarkable parallelism was demonstrated.

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[3] Chittenden, R. H., The nutrition of man, New York, F. A. Stokes Co., 1907.

[4] Chittenden, R. H., and Underhill, F. P., *Am. J. Physiol.*, 1917, **44**, 13.

[5] Goldberger, J., and Wheeler, G. A., *Bull. Hyg. Lab., U. S. P. H. S., No. 120*, 1920, 7.

[6] Wheeler, G. A., Goldberger, J., and Blackstock, M. R., *Pub. Health Rep., U.S. P. H. S.*, 1922, **37**, 1063.

[7] Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U. S. P. H. S.*, 1926, **41**, 297.

[8] Goldberger, J., and Wheeler, G. A., *Pub. Health Rep., U. S. P. H. S.*, 1928, **43**, 172.

[9] Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U. S. P. H. S.*, 1928, **43**, 657.

[10] Goldberger J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U. S. P. H. S.*, 1928, **43**, 1385.

[11] Denton, J., *Am. J. Path.*, 1928, **4**, 341.

A study was then made of the factors present in the foods shown to have prophylactic and therapeutic value and which were absent in the diet fed the animals. Investigation of the vitamin content of foods as far as known at that time threw suspicion on that factor known as water-soluble vitamin B. Experiment showed, however, that if materials known to be rich in vitamin B were heated to 120°C. for 1 hour the black tongue-preventing factor was still present. The heat treatment was sufficient to destroy the antineuritic power of the vitamin B complex.

This evidence indicated the presence in the effective material of a new factor named by Goldberger vitamin PP or "pellagra-preventing." This was found to be a factor necessary for the maintenance of growth and the prevention of dermatitis in young rats maintained on an otherwise basal ration. It was variously termed vitamin B_2 or G by other investigators. Yeast was found to be an excellent source of this accessory food substance. Finally, the feeding to human volunteers of a diet somewhat similar to that given the dogs resulted in the production of the symptoms of pellagra.

Wheeler,[12] in reviewing the subject, concluded that black tongue and pellagra were one and the same disease on account of their seasonal and geographical incidence, their common cause and similar course, their identical pathological changes, and their equal response to the same therapeutic and preventive measures.

Shortly after the publications of Goldberger and his colleagues, the subject of the etiology of black tongue was reopened by Underhill and Mendel.[13] They fed to dogs a diet similar to that used in their experiments already discussed. The principal ingredients were cracker meal, peas, and cottonseed oil. An acute disease marked by salivation, stomatitis, and diarrhea was produced, and the therapeutic effect of a variety of materials was tested. They concluded that butter and milk were effective both therapeutically and prophylactically, but that yeast was without potency. Furthermore, they felt that the anti-black tongue effect of butter and milk was subject to a seasonal variation, and that it depended upon the content of carotin. Finally, crystalline carotin was used to supplement the diet fed, and was shown to be effective in preventing and curing the experimental disease. The diet employed by this group of workers was so simple and so generally deficient that it is most difficult to analyze it in respect to any particular ingredient which might be lacking. The discrepancy between the results obtained by the two groups of investigators has never been satisfactorily explained. Neither group, however, apparently considered the similarity of the experimental disease to sprue and to pernicious anemia, and neither studied the associated blood changes. Furthermore, no animals are described in which a chronic pathological state was induced.

A third hypothesis concerning the etiology of pellagra and black tongue has been advanced by Bliss.[14] In a general survey of the diets eaten in regions where

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[12] Wheeler, G. A., *J. Am. Vet. Med. Assn.*, 1930, 77, 62.

[13] Underhill, F. P., and Mendel, L. B., *Am. J. Physiol.*, 1927–28, 83, 589.

[14] Bliss, S., *Science*, 1930, 72, 577.

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pellagra is prevalent, he concludes that they are deficient in iron content. Encouraging results are reported to follow the administration of iron in pellagra and in canine black tongue. From these facts, he evolved the hypothesis that both the canine and the human disease are manifestations of a dietary iron deficiency. In a personal communication, Bliss states that the hypothesis still remains neither proven nor disproven.

Stucky and Brand[15] reported experiments on rats in which symptoms of vitamin B_2 G deficiency appeared in spite of a diet containing an amount of iron sufficient to allow hemoglobin regeneration in young, anemic animals.

Methods

Dogs of good size, and of mongrel breed, largely short haired in type, were employed. The animals were kept under uniform conditions, in individual cages, with bedding of shavings. No special care was taken to avoid coprophagy. The diet was composed of the following ingredients: white corn-meal, 6000 gm.; California black eyed peas, 750 gm.; casein (leached—Casein Company of America), 900 gm.; cod liver oil, 225 cc.; cottonseed oil, 450 cc.; rice polishings, 600 gm.; calcium carbonate, 450 gm.; NaCl, 150 gm.

The corn-meal, peas, and casein were mixed and cooked for 2 hours in a steam cooker. The remaining ingredients were then added and thoroughly mixed. The dogs were fed daily and were allowed to eat as much as they chose.

The animals were weighed at weekly intervals, and blood was taken from the jugular vein in a standard amount of potassium oxalate for routine examinations. Counts were done more frequently when the condition of the animal seemed to warrant. Determinations of the numbers of erythrocytes and leukocytes were made in standard pipettes and counting chambers. The hemoglobin was estimated by the Sahli method with a glass standard. The Sahli tubes were carefully calibrated and checked at frequent intervals by the O_2-combining capacity method of Van Slyke.[16] The mean corpuscular volume was ascertained by the method of Wintrobe.[17] Smears of the blood were made on plain cover-slips and those covered with dried brilliant cresyl blue for the staining of reticulocytes.

In treatment, 200 gm. of raw lean beef were fed daily if the animal would eat. If not, a rice polishings concentrate prepared according to the method described by Evans and Lepovsky[18] was administered by stomach tube. Occasionally a brewers' yeast concentrate (Harris) was given. Successful therapeutic results were so difficult to obtain when the disease was at its height that it was often impossible to confine the treatment to some simple material.

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[15] Stucky, C. J., and Brand, E., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 1404.

[16] Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Volume II, Methods, Baltimore, The Williams & Wilkins Co., 1932, 337.

[17] Wintrobe, M. M., *J. Lab. and Clin. Med.*, 1931–32, **17**, 899.

[18] Evans, H. M., and Lepovsky, S., *J. Nutrition*, 1931, **3**, 353.

Prints of the dorsal surface of the tongue were made by applying a thin layer of ink with an ordinary library ink pad. Squares of soft paper were then lightly applied to the lingual surface with a rolling motion. With a little practice, excellent prints could be obtained. In the dogs a general anesthetic was usually employed.

General Results

Early experiments based on the feeding of the diet described, without the addition of rice polishings, were unsatisfactory since death usually occurred before blood changes could be demonstrated. Even with the addition of rice polishings, a substance extremely rich in vitamin B_1 and containing a certain amount of vitamin B_2 G (Evans and Lepovsky[18]), the incidence of acute, fatal disease was high. There appeared to be a striking variation in the ability of the individual animal to maintain life on the diet.

In a certain number of instances, however, a chronic disease developed in which well defined anemia was a feature, associated with a characteristic symptomatology. The averages presented in this publication are based on the findings in 10 dogs. Animals dying of intercurrent disease or from acute black tongue are not included. For the sake of simplicity only those protocols are presented which are fairly typical of the experimental disease.

In general the diet was well taken. Control animals which were given 4 gm. daily of liver extract (Eli Lilly and Company No. 343) ate no more than the dogs maintained on the unsupplemented diet, and remained in perfect health.

Animals which ate sparingly also failed to develop the disease under investigation, but usually died of intercurrent infections. It seemed clear that whatever symptoms developed were due to the absence of an accessory food factor in the diet and not to lack of salts, metallic elements, particular proteins, or caloric intake.

Symptomatology

Certain symptoms, which have not been described as occurring in ordinary acute black tongue, were manifested by the animals which developed the chronic recurrent disease. These symptoms will be described in detail, since upon their presence depends the similarity of the experimental condition produced to disease syndromes in human beings.

The stomatitis of acute black tongue appears first as localized irregular patches of injection on the floor of the mouth, and on the labial and buccal mucous membranes. This extends until the entire oral mucous membrane is deeply injected and shows a superficial desquamation. In the dogs which ran a chronic course, the mucous membrane changes tended to be more generalized and less acute in character. About 6 weeks after the experimental diet feeding was begun, occasional irregular patches of injection appeared on the surfaces of the labial and buccal mucous membranes. Instead of progressing rapidly to necrosis, the entire lining of the oral cavity frequently became a deep, dusky red in color, and presented a peculiar and striking dry, velvety, rather granular appearance. This change often persisted for many days or even weeks. From time to time, discrete, yellowish, slightly raised aphthae appeared, some of which regressed while others went on to circumscribed desquamation of the epithelium.

Glossitis was a marked feature and was strikingly similar to that seen in sprue and pernicious anemia in human beings. In acute black tongue, the glossitis appears late, is usually confined to the margins of the tongue and is quite extensive, often being almost gangrenous in nature. In the chronic recurrent conditions under discussion, however, the glossitis was frequently widespread, milder in degree, and at times completely unassociated with stomatitis. The first manifestation was a loss of papillae along the margins of the tongue with a faint, smooth, pink to red coloration (Figs. 1 to 3). The involved area was glossy, smooth, and glistening. As the condition progressed, the color became a deeper red, and the area involved in the atrophic process more extensive. Finally, a large part of the dorsum of the tongue might be a deep, fiery red color with complete loss of papillae. Changes in the atrophic areas took place with astonishing rapidity, particularly as healing set in. Large areas of new papillae frequently appeared overnight, and there was a well defined impression that the depth of color might vary from hour to hour. Exactly this same phenomenon has been observed in cases of human sprue. Figs. 4 to 7 show prints taken from the tongues of two dogs at the height of the glossitis and again after regeneration of papillae had taken place. Fig. 8 is a photograph of a tongue presenting advanced atrophic glossitis.

The manifestations of disordered function of the gastrointestinal tract were two: anorexia and diarrhea. In the animals which ran a chronic recurrent course, the appetite was in general well maintained. Only when the salivation and stomatitis were at their height was the diet refused entirely. From time to time, however, periods of several days would elapse during which little food was taken. The diarrhea was variable in character. During an acute exacerbation, it was usually profuse, watery, and brown in color. Blood was frequently passed. In animals running a more protracted course, periods of several days during which the stools were semisolid to liquid, yellow in color, and voluminous would alternate with periods in which normal stools were passed. Cultures of the diarrheal stools on Sabouraud's medium gave growths of yeast-like organisms in about 10 per cent of the attempts. Control animals never showed such organisms. No attempt was made to identify and classify the type of yeast obtained.

Loss of weight was a striking feature. This again is rarely encountered in studying acute black tongue. The average loss was about 20 per cent of the original weight. That this loss was due to some specific deficit in the diet is shown, as previously mentioned, by the fact that control animals eating the same diet supplemented by 4 gm. daily of liver extract No. 343 maintained their original weight throughout the experiment.

The Anemia

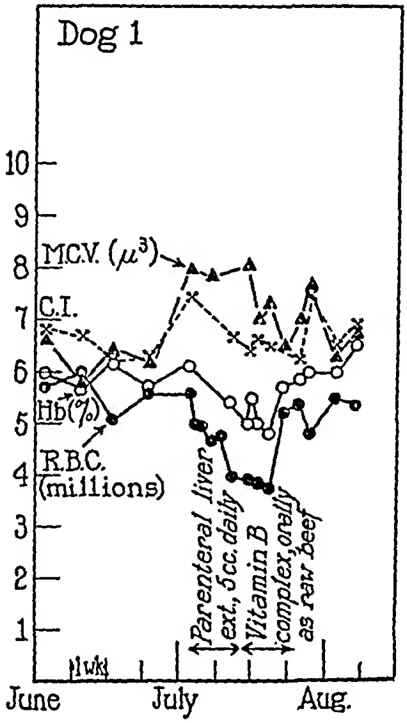
The average maximum variation in blood values observed in the dogs reported are presented in Table I. During the control period before the diet feeding was begun, the average red cell count was 6,440,000 and the average hemoglobin level, 87.7 per cent. At the low point of the anemia, the average erythrocyte count was 3,100,000, and the average hemoglobin level, 56.4 per cent. There was an average decrease in erythrocytes of 3,340,000, and in hemoglobin of 31.3 per cent. There was in addition an average maximum increase in color index of 22.5 points, and in mean corpuscular volume of 22.9 cubic microns. From the figures it is apparent that the average maximum decrease in numbers of erythrocytes was greater than the average decrease in hemoglobin and was associated with an increase in both color index and mean corpuscular volume. These findings are characteristic of the macrocytic anemias of sprue and pernicious

TABLE I

The Average Maximum Variations in Blood Values of 10 Dogs with Chronic Black Tongue and Anemia

	Normal	Low point of anemia	Difference	Per cent difference
Average red cell count	6,440,000	3,100,000	3,340,000	53
" hemoglobin, *per cent*	87.7	56.4	31.3	35.6
" color index	0.685	0.91	22.5	34.5
" mean corpuscular volume, *cubic microns*	60.2	83.1	22.9	33.6

anemia in man. It should be remembered that the variations in blood values discussed were maximum and that they were not present throughout the period during which the animals were under observation. There was a striking tendency to spontaneous remissions and exacerbations of the anemia. There was at no time a marked increase in the pigment content of the plasma. In a few cases in which blood and plasma volume determinations were done by the vital red method of Rowntree no considerable variations were encountered. Fluctuations in the numbers of leukocytes will be described in another communication. Examinations of the blood of control animals kept under similar conditions failed to show significant variations from normal levels.



TEXT-FIG. 1

PROTOCOLS

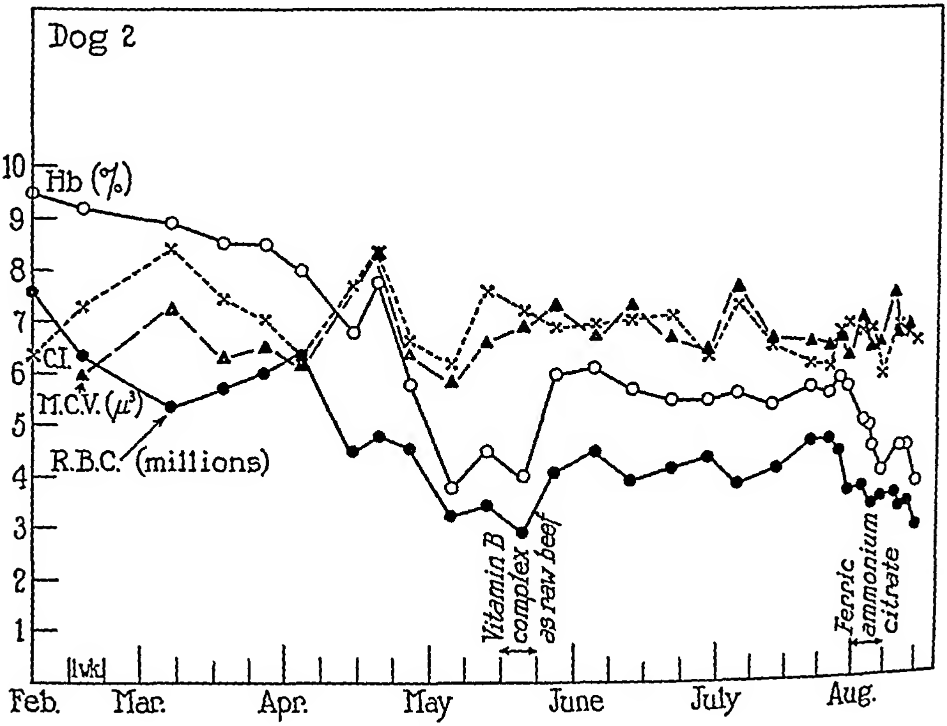
Dog 1.—Terrier-hound cross. Text-fig. 1. 3–22–32. Weight 10.3 kilos. R. B. C. 5,540,000. Experimental diet feeding begun. Gastric analysis showed free hydrochloric acid in all samples. 4–21–32. Weight 10.9 kilos. R. B. C. 5,500,000. Salivation was present with mild generalized injection of buccal mucosa, anorexia, and atrophy with redness of the lateral borders of the tongue. Copious watery diarrhea was present. 400 gm. of meat were fed. 4–25–32. Weight 10.3 kilos. R. B. C. 5,930,000. Recovery followed. 5–21–32. Weight 11 kilos. R. B. C. 4,700,000. Diarrhea, salivation, and mild generalized injection of the buccal mucous membrane were present. The stools were voluminous and yellow in color. Fluids were given intravenously and intraperitoneally with 100 cc. of alcoholic extract of rice polishings. Improvement occurred in 4 days. 6–7–32. Weight 10 kilos. R. B. C. 5,000,000. Active stomatitis with the formation of a thin necrotic membrane was present. Salivation and diarrhea were copious, with marked redness and atrophy of the lateral borders of the tongue. Physiological saline solution was administered intravenously and intraperitoneally. Rice polishings concentrate was given orally. Improvement followed in 4 days. 7–7–32. Weight 8.7 kilos. R. B. C. 4,900,000. Fourth attack, with marked, generalized stomatitis, great salivation, and active, yellow diarrhea. Marked atrophy of the tongue was present. The administration of physiological saline solution intravenously, and of rice polishings extract orally, was followed by improvement in 6 days.

After this attack there was a sharp fall in the blood values to a low erythrocyte count of 3,740,000 cells per c.mm. The color index was somewhat greater than normal, and there was a slight increase in the mean corpuscular volume. The course of the blood changes is shown in Text-fig. 1. Liver extract (Lilly No. 343) prepared for parenteral injection was administered intramuscularly each day for 10 days without improvement in blood values.

7–18–32. 100 gm. of raw beef were fed daily in addition to the experimental ration. There was a prompt return of the blood values to a high level.

This animal had four acute attacks of stomatitis and gastrointestinal disturbance. Following the last attack anemia developed. No improvement in blood values followed the parenteral injection of liver extract but striking improvement followed meat feeding. In the interim between acute attacks, the buccal mucous membrane of this animal presented a dry, irregular, deep red, granular appearance. A variable degree of lingual atrophy was constantly present with exacerbations during acute attacks. No decrease in the ability of the stomach to secrete hydrochloric acid was observed.

Dog 2.—Great Dane-bull cross. Text-fig. 2. 2–6–32. Weight 20.5 kilos. R. B. C. 7,500,000. The experimental diet feeding was begun. Gastric analysis showed free hydrochloric acid and ferments in all samples. 3–7–32. Weight 20.7 kilos. The animal was inactive and salivating mildly. Patchy reddening of the mucous membrane of the upper lip was present. 3–8–32. Weight 20 kilos. R. B. C. 5,300,000. Extensive uniform injection of the buccal and labial mucous membrane had developed, and salivation was marked. Diarrhea was active with liquid, brown, watery stools. 3–12–32. Weight 20 kilos. R. B. C. 5,700,000. A general diffuse redness of the buccal mucous membrane was present with a rather dry, granular appearance. Nearly all the stools after 3–7–32 were watery in consistence and ranged from brown to yellow in color. This condition persisted until 3–24–32. 3–24–32. Weight 18.9 kilos. R. B. C. 6,000,000. The lateral margins of the tongue showed marked atrophy of papillae with a striking purplish red color. An illustration of the tongue at this stage is shown in Figs. 3 and 8. The mouth and tongue changed very little until 4–2–32. The diarrhea persisted. 4–2–32. Weight 18.9 kilos. R. B. C. 4,490,000. The margins and tip of the tongue had become glass-smooth and a fiery red in color. A print of the tongue at this stage is shown in Fig. 5. There was the faintest trace of injection of the buccal mucous membrane. The dog was eating the diet well and was in good condition. 4–5–32. Weight 18 kilos. R. B. C. 4,400,000. The fiery red color of the tongue was rapidly fading, and new papillae were appearing in the areas which had been completely atrophic (Fig. 4). Otherwise the mouth showed no abnormality. Loose voluminous yellow stools were the rule, but occasional brown, formed stools were passed. 4–12–32. Weight 17 kilos. R. B. C. 4,790,000. In the interim there was little change. On this date the margins

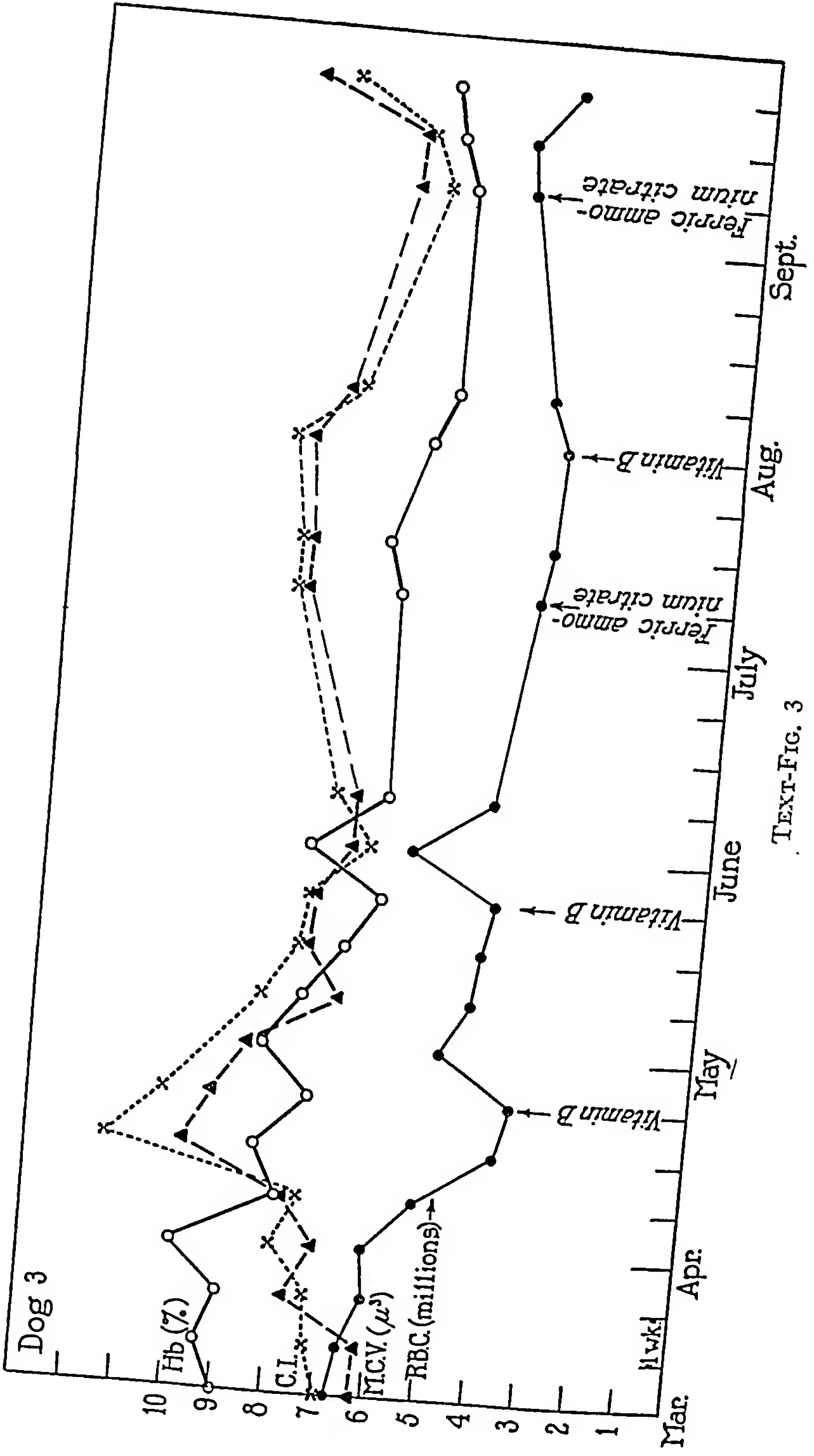


TEXT-FIG. 2

of the tongue were once more smooth, atrophic, and a fiery red in color. The stools were large, yellow, and semifluid to fluid. From these stools a yeast-like organism was grown on Sabouraud's agar. The animal was very weak and listless. 4-20-32. Weight 15 kilos. R. B. C. 4,500,000. The bright red injection of the lingual borders had faded, and new papillae were appearing. The diarrhea was much less but still present intermittently. 200 gm. of raw, lean beef were added to the diet daily for 10 days. Within a few days the lingual inflammation and atrophy had disappeared, the diarrhea had ceased, and the animal gained rapidly in health and strength. There was an improvement in the blood levels. From this point on there was a steady fall in the number of erythrocytes to a low level of just under 3,000,000. The changes in the other values are graphically shown in Text-fig. 2. 7-5-32. Weight 14 kilos. R. B. C. 3,600,000. The tongue was once more atrophic, and presented a bright red coloration of the margins. Diarrhea was intermittent. The stools were bulky, yellow, and fluid to semifluid in consistence. Ferric ammonium citrate, 1 gm. daily, was added to the diet. The condition of the tongue and gastrointestinal tract persisted unchanged to 8-10-32, when the animal was found dead. The pathological changes will be described elsewhere.

This animal showed chronic, recurrent, atrophic glossitis and gastrointestinal disturbance for a period of 6 months. As seen from Text-fig. 2, there was an anemia of advanced degree which was improved by feeding meat, and did not respond to iron administration. The pronounced, necrotic mucous membrane lesions so commonly seen in acute black tongue were not encountered in this instance. The extent and marked character of the lingual lesions may be seen in the tongue print, Fig. 5, the photograph, Fig. 8, and in the colored Fig. 3. There was a marked loss of weight. Despite the striking gastrointestinal symptomatology, there was at no time a decrease or loss in the ability of the stomach to secrete free hydrochloric acid. The bone marrow at autopsy was extremely red and hyperplastic.

Dog 3.—Black and tan terrier. Text-fig. 3. 3-9-32. Weight 16.1 kilos. R. B. C. 6,700,000. Experimental diet feeding begun. Gastric analysis showed free hydrochloric acid in all specimens. 4-20-32. Weight 14.8 kilos. R. B. C. 3,300,000. The food was refused, and pronounced, watery diarrhea was present. Multiple red patches were present on the buccal mucous membrane. The dog was fed 200 gm. of raw beef daily for 5 days, when the mouth became free from lesions, and the appetite returned. There was an improvement in the blood values. 5-27-32. Weight 15.1 kilos. R. B. C. 4,440,000. Copious, watery diarrhea was present. A mild, patchy injection of the buccal mucosa had appeared. 5-28-32. Weight 15.6 kilos. R. B. C. 4,400,000. Marked redness and atrophy of the

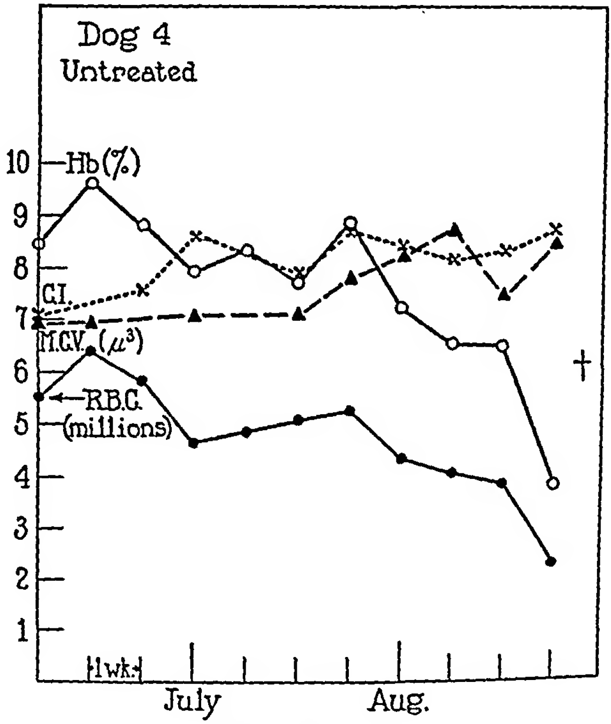

Dog 3
Hb (%)
C.I.
M.C.V. (μ^3)
RBC (millions)→
1 wk.
10
9
8
7
6
5
4
3
2
1
Mar.
Apr.
May
June
July
Aug.
Sept.
Vitamin B
Vitamin B
Ferric ammonium citrate
Vitamin B
Ferric ammonium citrate

TEXT-FIG. 3

tongue margins had developed and the papillae had entirely disappeared (Figs. 1, 2, and 7). A mild diarrhea with bulky yellow stools was present. The dog was inactive. This condition continued for 4 weeks. There were variations in the intensity of the atrophic glossitis, but at no time did more than a few papillae return. Cultures of the stools on Sabouraud's medium were repeatedly positive for yeast-like organisms. Meat, 200 gm. daily, was fed for 4 days, with temporary improvement in blood levels. 6–23–32. Weight 14.2 kilos. R. B. C. 3,800,000. The tongue atrophy had now begun to disappear, and many new papillae were present (Fig. 6). The buccal mucous membrane presented a dry, granular appearance. Except for persistent diarrhea the animal was in good condition and eating well. 7–5–32. Weight 12.6 kilos. R. B. C. 3,800,000. In the interim there was little change. Loose, yellow stools containing yeast were passed nearly every day. The tip and borders of the tongue were smooth and a deep pink in color. Ferric ammonium citrate was administered, 1 gm. daily, for 2 weeks without improvement in blood values, glossitis, or gastrointestinal symptoms. There was a slow but constant increase in the activity of the symptoms. 7–30–32. Weight 11 kilos. R. B. C. 3,500,000. The animal appeared unusually quiet. The glossitis was marked. 200 gm. of raw beef were fed daily for 6 days followed by striking improvement. The redness of the tongue faded and papillae reappeared. The diarrhea ceased for a few days. 8–14–32. Weight 10.7 kilos. R. B. C. 3,700,000. The diarrhea began once more, and mild atrophic glossitis developed. The symptoms continued until 9–24–32 when meat was again fed and the symptoms disappeared. There was a rise in blood values. 10–10–32. Weight 14 kilos. R. B. C. 5,850,000. The buccal mucosa again took on the red, dry, granular appearance described previously. An intermittent diarrhea with bulky yellow stools began. The tongue was very red and the papillae were completely absent on the tip and borders. Ferric ammonium citrate, 1 gm. daily, was administered for 10 days without effect on either symptoms or blood. 10–16–32. Weight 16 kilos. R. B. C. 5,200,000. The buccal mucous membrane was very injected, and a large number of yellow aphthae were present. Atrophic glossitis was clear-cut. The diarrhea was active, and the stools contained blood. 10–26–32. Death occurred.

This animal, like Dog 2, ran a prolonged, chronic course with pronounced glossitis, persistent diarrhea, and a marked anemia. As may be seen from Text-fig. 3, the blood showed cycles of macrocytosis relieved by meat feeding. No effect of liver extract (Lilly No. 343) injected intramuscularly was observed, nor was iron in large doses potent in relieving the symptoms or in bringing about improvement in blood values. At no time was any decrease in the hydrochloric acid content of the stomach noted. The femoral bone marrow was extremely red and hyperplastic. There was a maximum weight loss of 5.4 kilos.

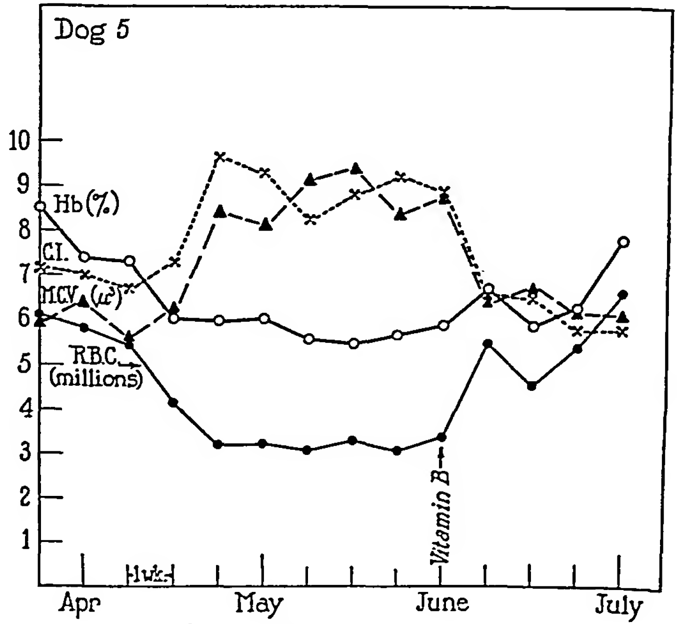
Dog 4.—Short haired shepherd. Text-fig. 4. 6–14–32. Weight 17 kilos. R. B. C. 6,400,000. Experimental feeding was begun. Gastric analysis showed free hydrochloric acid in all samples. The diet was well taken, and no abnormalities of the mouth were noted for 8 weeks. As shown in Text-fig. 4, there was a progressive pronounced drop in the blood values, and a steadily increasing macrocytosis. A dry, injected, oral mucous membrane was constantly present. 9–2–32. Weight 14 kilos. R. B. C. 2,260,000. The tip and lateral margins of the tongue were completely atrophic and a brilliant scarlet in color. Diarrhea was present. Liver extract No. 343 was injected intravenously and intramuscularly with large amounts of physiological saline solution. 9–3–32. The animal was prostrate. The therapy was repeated, but death ensued on the following day.



TEXT-FIG. 4

This animal, in contrast to the three previously described, showed a maximum of hematological effect and only terminal lingual and gastrointestinal symptoms. This difference in the clinical course must be ascribed to individual variations in the experimental animal. No treatment was administered until late in the disease. Comparison of the chart of this animal's course with those of the treated dogs shows strikingly the effect of early therapy with material rich in vitamin B_2 G on the experimental disease.

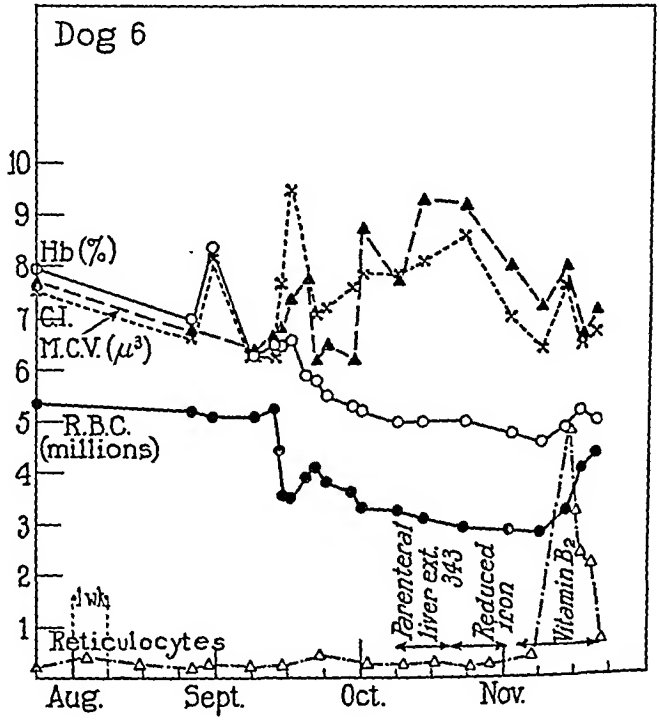
There was a maximum weight loss of 3.6 kilos. The bone marrow at autopsy was not red and hyperplastic in this instance, a fact in keeping with the short course and rapid fall in blood values. There was no decrease in the ability of the stomach to secrete hydrochloric acid.



TEXT-FIG. 5

Dog 5.—Black shepherd. Text-fig. 5. 1–17–32. Weight 17.9 kilos. R. B. C. 6,000,000. Experimental diet feeding begun. Free hydrochloric acid was present in all specimens of gastric contents. 3–11–32. Weight 10.2 kilos. R. B. C. 4,100,000. A mild, patchy injection of the buccal mucous membrane was present. A slow spread of the lesions took place until 3–22–32 when they disappeared spontaneously. 3–29–32. Weight 16.4 kilos. R. B. C. 3,200,000. The macrocytic anemia continued to be present. A few patches of redness remained in the mouth, and the dog was rather inactive. An intermittent diarrhea had set in with soft, bulky, yellow stools. 4–24–32. Weight 14.8 kilos. R. B. C. 3,100,000. In the interim there were a persistent diarrhea and generalized velvety injection of the buccal mucous membrane. Atrophy of the tongue margins appeared and increased in intensity. The animal was inactive. Cultures of stools on Sabouraud's agar were repeatedly positive for yeast-like organisms. Autolyzed yeast, 30 gm. daily, was given by mouth followed by a prompt amelioration of symptoms and improvement in blood values.

Although this dog was fed the experimental diet for a long period after the improvement in blood values shown in the chart, no further anemia developed. Death in an attack of acute black tongue terminated the experiment. The only possible explanation of this sequence of events in the light of present knowledge is that a variable individual factor predisposing to anemia is required. This occasional relative independence of oral symptoms and anemia is not uncommonly encountered in dealing with sprue and pernicious anemia in human beings. There was a maximum weight loss of 4 kilos. No decrease in the acidity of the gastric contents was noted.



TEXT-FIG. 6

Dog 6.—Mongrel terrier. Text-fig. 6. 7–26–32. Weight 15 kilos. R. B. C. 5,380,000. The experimental diet feeding was begun. 9–3–32. Not weighed.

A diffuse redness of the buccal mucous membrane was observed for the first time. Bulky, semisolid to liquid, yellow stools were passed almost daily. 9–15–32. Weight 12.6 kilos. R. B. C. 3,500,000. Profuse yellowish diarrhea was present daily in the interim. The tongue showed a very striking redness and marginal atrophy. A mild generalized injection of the buccal mucosa was present. Stool cultures were repeatedly positive for yeast-like organisms. 10–8–32. Weight 11.8 kilos. R. B. C. 3,100,000. Some papillae had reappeared on the tongue and the redness had largely faded. The diarrhea decreased. Liver extract No. 343 (Lilly) was injected daily in 5 cc. amounts. As seen from the chart no improvement in blood values resulted. 10–18–32. Weight 11.2 kilos. R. B. C. 2,900,000. The atrophic glossitis and gastrointestinal manifestations were improved. The administration of reduced iron, 1 gm. daily, for 10 days was begun. 10–28–32. Weight 11.0 kilos. R. B. C. 2,900,000. No improvement of blood values had occurred, and iron therapy was discontinued. Atrophy and redness of the tongue borders were present with a moderate injection of the buccal mucous membrane. 11–1–32. Weight 10.5 kilos. R. B. C. 3,280,000. Autoclaved brewers' yeast autolysate (Harris) was given, 15 gm. daily for 10 days. A mild reticulocyte rise and improvement in blood values resulted in an erythrocyte count of 4,370,000.

After the routine feeding of the experimental diet was reinstated, this animal died in an acute attack of black tongue without marked anemia.

The course of this animal's illness is particularly important since three methods of therapeusis were employed, and all of the clinical manifestations under discussion were present. The lingual atrophy and redness were striking and present nearly constantly for a long period. The diarrhea was yellow, voluminous, and persistent. Cultures on Sabouraud's agar repeatedly were positive for yeast. There was a macrocytic anemia of marked degree which failed to improve upon the administration of iron or of injected liver extract No. 343 (Lilly). A reticulocyte rise, a drop in color index and mean cell volume, and a rise in erythrocyte numbers and hemoglobin content occurred when material rich in vitamin B_2 was fed. During the time the animal was under observation, there was a loss of weight of 4.65 kilos.

DISCUSSION

From the findings presented, it appears that a chronic recurrent disease in dogs was produced by the feeding of a particular diet. The disease was marked by chronic stomatitis with acute exacerbations, by salivation, by prolonged and marked atrophic glossitis, loss of weight, diarrhea, and, most important, anemia. The anemia was moderately severe, bore a relationship to the duration and intensity of the concurrent symptomatology, and was at times strikingly macrocytic in character. Certain factors in this experimental disease deserve special consideration.

The exact etiology of the condition produced still remains obscure. The diet fed was selected because it could be relied upon to produce the cardinal oral symptoms desired. It was supplemented with rice polishings to rule out the possibility that a deficiency in antineuritic vitamin B_1 was present. This supplement undoubtedly contained a small amount of vitamin B_2 G. The prophylactic and therapeutic effect of materials rich in vitamin B_2 G, such as meat, rice polishings concentrate, and autoclaved yeast, was striking. These facts make a strong argument for the hypothesis that vitamin B_2 G or some closely related substance was the missing factor in the diet. That vitamin can be employed only in an extremely crude form. Titration of the diet fed for its power to cause growth in young rats has not yet been carried out; hence an even approximately exact statement as to the absent factor cannot be made. It seems clear that lack in vitamin A or antineuritic B_1 was not at fault. The same diet supplemented with liver extract powder (Lilly No. 343), 4 gm. daily, maintained the animals in perfect health. This material is almost free of salts and of protein (Cohn[19]). It is rich in vitamin B complex (Guha[20]). Hence it appears that the diet contained no material which was toxic *per se* and was not deficient in particular proteins or in mineral constituents. Carotin in large doses parenterally administered was not therapeutically effective nor was iron ammonium citrate. Lack of these two materials cannot be considered causative. Pending further experimentation, lack of some substance closely associated with vitamin B_2 G seems to be the most probable etiology.

Variability in the response of dogs kept under the same experimental conditions was striking. No explanation of this fact is at hand other than a constitutional variation in the host. A similar lack of uniformity is found in the human response to particular dietary de-

[19] Cohn, E. J., McMeekin, T. L., and Minot, G. R., *J. Biol. Chem.*, 1930, 87, p. xlix.

[20] Guha, B. C., *Lancet*, 1931, 1, 864.

ficiencies. Most of the animals eventually died in an attack of acute black tongue even when the diet was supplemented with rice polishings. Great patience was required to obtain a convincing series of animals presenting a more or less chronic disease.

The criticism may well be advanced that the changes in cell volume reported might have been the result of slight changes in tonicity of the blood plasma, themselves the result of dehydration due to diarrhea. That possibility seems unlikely since no consistent correlation between diarrhea and change in cell size was observed. Indeed, the presence of frequent liquid stools was usually associated with a rise in the blood count and a decrease in the average cell size. Furthermore, loss of water from the intestinal tract should result in an increased tonicity of plasma and a decrease in cell size, rather than the reverse.

At the outset of the experiment, it was thought possible that a loss of the ability of the stomach to secrete hydrochloric acid would be encountered. This only occurred rarely, as a terminal event, and will be described elsewhere. Indeed, those animals which ran the most chronic course were those which maintained their gastric secretory function.

The changes in the bone marrow of the animals autopsied at the height of the anemia were frequently striking and characteristic. The histological details will be presented in a subsequent communication. It suffices to state here that the femoral marrow was the site of intense cellular activity with nearly complete absence of fat and replacement by young hematopoietic cells. In many respects this marrow change was similar to that seen in severe sprue (Rhoads and Castle[21]) and in pernicious anemia (Peabody[22]). There was a change in the extent of active marrow as compared with the femoral marrows of normal dogs and of the experimental dogs upon which biopsies were performed before the feeding of the experimental diet was begun. There was also a change in the cell type of the active marrow. This was best seen by comparing the histology of normal active vertebral marrow which was predominantly normoblastic with the pathologically active femo-

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[21] Rhoads, C. P., and Castle, W. B., *Am. J. Path.*, in press.

[22] Peabody, F. W., *Am. J. Path.*, 1926, 2, 487.

ral marrow of anemic dogs which was predominantly megaloblastic in type.

A striking fact was the failure to demonstrate an improvement in symptoms and blood values following the parenteral administration of large doses of liver extract. Three instances of this fact are included in the individual protocols. Twelve other trials of parenteral liver extract as a therapeutic agent were followed by similar failure. Moreover, the daily administration of that amount of liver extract derived from 50.0 gm. of whole liver prepared for parenteral administration injected intramuscularly each day failed to prevent the development of anemia and glossitis. That this material is specific in relieving the symptoms of sprue and pernicious anemia has been conclusively shown. Since the oral administration of substances rich in vitamin B_2 G did produce improvement in blood values, it would appear that the dog utilizes vitamin B as such in hematopoiesis and not as an intermediate product of gastric digestion as does the human being. Further experiments on this point are in progress.

No consideration of anemia in the dog should fail to include the possibility of infection with *Bartonella canis* (Kikuth[23]). Examination of blood smears from the animals included in this report failed to reveal *Bartonella* bodies. That an infection with the organism may be latent and still depress the blood values under abnormal conditions is quite possible. It can only be stated that in the experiments no evidence of such an infection was found.

SUMMARY AND CONCLUSIONS

1. By the feeding of a particular diet, apparently lacking a substance closely associated with vitamin B_2 G, a chronic disease may be produced irregularly in dogs.

2. The disease is characterized by atrophic glossitis, diarrhea, loss of weight, and anemia.

3. The disease can be prevented and relieved by materials rich in vitamin B_2 G.

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[23] Kikuth, W., *Centr. Bakt., 1. Abt., Orig.*, 1929, **113**, 1.

EXPLANATION OF PLATES

PLATE 30

FIGS. 1 to 3. Paintings of the tongues of dogs with chronic black tongue showing various degrees of injection and atrophy of papillae.

PLATE 31

FIGS. 5 and 7. Prints taken from the dorsal surfaces of the tongues of dogs with the atrophic glossitis of chronic black tongue.

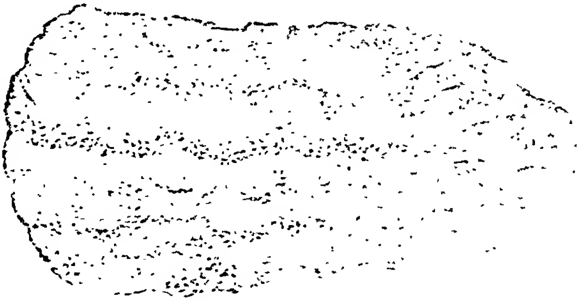
FIGS. 4 and 6. Prints from the same tongues after recovery had taken place.

PLATE 32

FIG. 8. Photograph of the tongue of a dog with chronic black tongue showing atrophy of the papillae of the lateral lingual borders.

1

2



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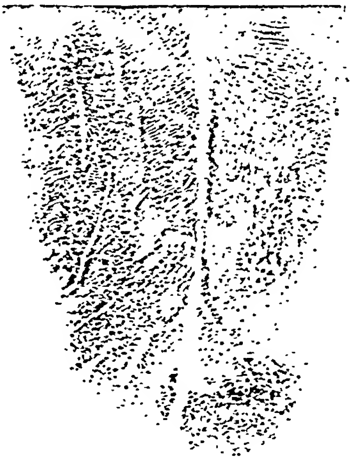
(Rhoads and M[illegible]: C[illegible])



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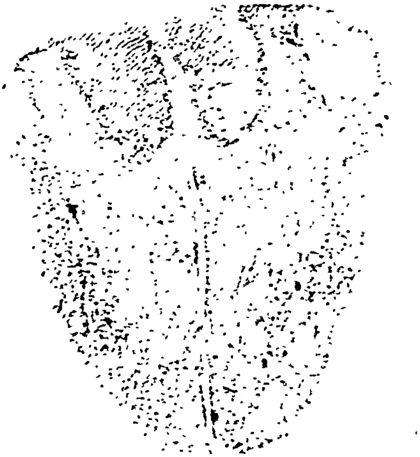

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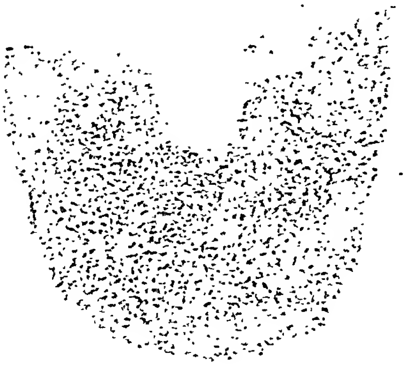

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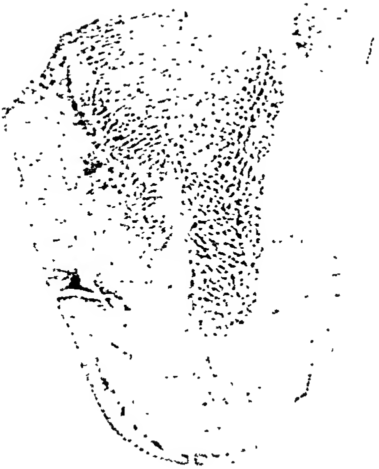

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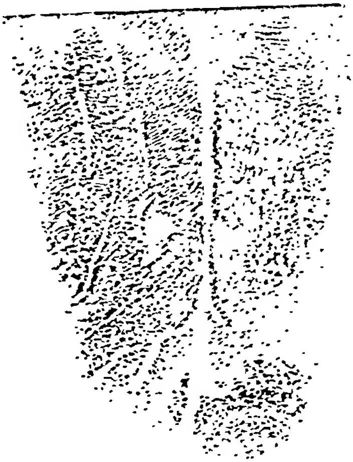
(Rhoads and Miller: Chronic black tongue with anemia)



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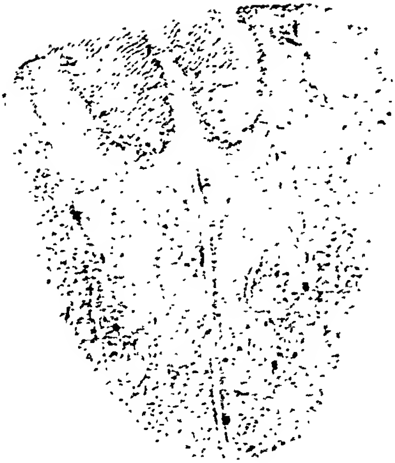

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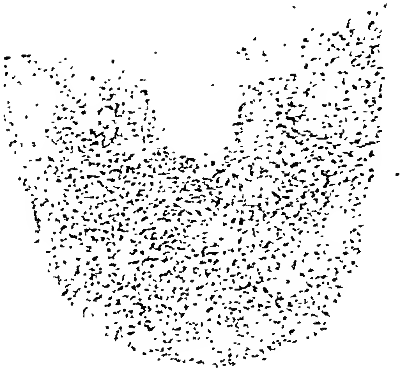

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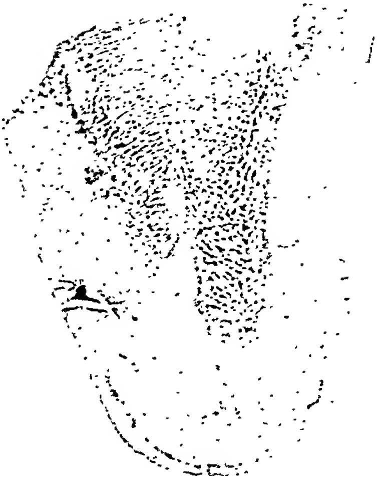

7

(Rhoads and Miller: Chronic black tongue with anemia)

8

Photographed by Louis Schmidt

(Rhoads and Miller: Chronic black tongue with anemia)

INFECTIOUS PAPILLOMATOSIS OF RABBITS

By RICHARD E. SHOPE, M.D.

With a Note on the Histopathology

By E. Weston Hurst, M.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

Plates 33 to 35

(Received for publication, July 19, 1933)

Our attention was recently called to a disease occurring in wild cottontail rabbits in northwestern Iowa.[1] Rabbits shot there by hunters were said to have numerous horn-like protuberances on the skin over various parts of their bodies. The animals were referred to popularly as "horned" or "warty" rabbits.

Warts from a naturally occurring case of the disease in Iowa were obtained and sent to the laboratory in sterile 50 per cent glycerol. These glycerolated warts furnished us our original material for investigation. A little later, in a shipment of a dozen wild cottontail rabbits from southern Kansas, three were found to be affected with the same wart-like disease. To date, out of 75 wild cottontail rabbits received from Kansas eleven have been found to carry one or more warts. These eleven animals serve as the basis for our description of the naturally occurring disease.

Description of the Naturally Occurring Disease

In wild cottontail rabbits the presence of warts has caused no apparent discomfort in our experience and induced no demonstrable evidence of generalized illness. Most of the animals were sacrificed, shortly after their arrival, for pathological material, but four, kept under observation for 7 weeks or longer, at no time appeared ill and were in good physical condition when finally killed. The number

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[1] We are grateful to Mr. T. A. McKichan of Cherokee, Iowa, who first told us of the disease, and to Mr. Clifford Peck of Cherokee and Mr. Earl Johnson of Rago, Kansas, who furnished us with naturally occurring cases of the disease.

of warts on individual animals in our series varied from one to ten in all cases except one. The exceptional animal was almost literally covered with warts and these, when removed at autopsy, were sufficient to fill a 200 cc. flask. The most common sites for the warts were on the inner aspect of the thighs, the abdomen, or about the neck and shoulders. Individual warts varied somewhat in size but the greater number were from 0.5 to 1 cm. in diameter and from 1 to 1.5 cm. in height. They were black or grayish black in color, well keratinized, and the upper surfaces were frequently irregular or fissured. They were roughly oval in shape when viewed from the side (Fig. 1). The bases, in most instances, were narrower than the mid-portion. The lateral surfaces of the warts appeared vertically striated because each individual wart was composed of closely packed and almost homogeneous vertical strands of tissue. On cut section, an average wart had a white or pinkish white fleshy center, and the upper portion and its lateral surfaces were grayish black and keratinized. The vertically striated structure was particularly evident on cut section. Attachment to the skin was loose as evidenced by the ease with which warts were knocked or pulled off when animals were handled. Warts removed in this way left a rather freely bleeding surface which in most instances healed without complication; sometimes a second wart appeared at the same site.

Experimental Transmission

No difficulty has been encountered in transmitting the condition to either domestic or wild cottontail rabbits when materials from naturally occurring cases have been employed. The method used is, in brief, as follows:

Either freshly removed warts or those that have been stored in 50 per cent glycerol at refrigerator temperature are ground to a fine paste with sterile sand and physiological saline in a mortar. More physiological saline is added to make a 3 to 5 per cent final suspension. Such a suspension is then centrifuged and the supernatant fluid, which is only slightly turbid, is removed and used for inoculation. Suspensions prepared in this way remain infectious for at least a month when kept at refrigerator temperature.

Inoculation by scarification was regularly performed in these experiments. Rabbits to be inoculated were shaved on the abdomen or sides and lightly scarified either by needle or by rubbing the shaven skin with a moderately coarse grade of sterilized sandpaper. To obtain discrete warts the former method was employed, while scarification with sandpaper was used when a confluent and massive growth of warts was desired. The scratches were made only deep enough to cause a barely perceptible oozing of blood-tinged fluid. A small amount of the infectious suspension was immediately applied by dropping it from a syringe, and this fluid was rubbed well into the scarifications by means of a spatula or the flat handle of a scalpel. The area thus inoculated was allowed to become almost dry before the animal was released and put into its cage.

Course of the Experimental Infection

The disease produced by experimental infection of either wild cottontail or domestic rabbits followed a typical course when infectious material from naturally occurring cases was employed. The period of time elapsing between inoculation and the first appearance of macroscopically detectable lesions varied from 6 to 12 days with an average of slightly more than 8 days. This variation in the incubation period was probably due more to differences in the potency of the infectious suspensions than to differences in the resistance of individual animals, for when the same suspension was employed in inoculating a series of rabbits the incubation period was the same in the entire series.

The first detectable lesions consisted in minute, barely visible elevations along the lines of scarification. These, on the 1st day, appeared macroscopically to be tiny vesicles, an appearance not supported by histological examination. By the 4th or 5th day the lesions were more definite and numerous and were usually pink in color. They were approximately 1 mm. in diameter and height and had lost their delicate vesicular appearance. From this stage on, growth was constant although its rapidity varied considerably. From the 16th to the 20th day after their first appearance, the lesions were approximately 3 mm. in diameter and height if they were isolated on the shaven areas or, if confluent, they constituted a more or less solid mass of rough wrinkled pinkish keratinized tissue 3 to 4 mm. in thickness. They had, by this time, acquired a definitely warty appearance, their surfaces were keratinizing, and their sides exhibited the peculiar type of perpendicular striations seen in the naturally occurring warts. The warts, whether separate or confluent, continued to increase in size for an indefinite period, and as the lesions became older they became more and more cornified until finally the upper portions were very hard. The lower portions, however, usually remained fleshy to the touch. At 6 weeks, individual warts or confluent masses were from 1.2 to 1.5 cm. in height; the skin on the portion of the body upon which they were developing had become pendulous and was thrown into large stiff folds. Animals sacrificed at this time exhibited an enormously increased blood supply in the subcutaneous tissue underlying the warts. In spite of the great size of many of our experimentally produced wart masses, the animals showed no loss in weight and the entire course of the disease was free from any general clinical evidence of illness. In their gross appearance the experimental warts in both domestic and wild rabbits have been identical with those seen in the naturally occurring disease. Photographs of experimentally produced warts are given in Figs. 2 to 4.

Experimental warts, as well as those occurring naturally, appear to remain stationary when they reach 1 to 1.5 cm. in height. One of our rabbits, however, at present, 6 months after inoculation, is carrying a large wart mass which in places is 3 cm. in height. With two exceptions, we have seen no warts retrogress in animals infected in the usual way. In the exceptional animals, one a wild and the other a domestic rabbit, warts developed slowly after an unusually long incubation period. They reached a maximum height of only 2 to 3 mm. between 30 and 40

Filtrability of the Wart-Inducing Agent

Warts to be used as a source of infection in the filtration experiments were removed from the 50 per cent glycerol in which they had been stored and were washed in three changes of sterile physiological saline. They were then minced with sterile scissors, ground in a mortar with sterile sand, and suspended in sufficient physiological saline to make an approximately 5 per cent suspension. Suspensions thus prepared were cleared by centrifugation. The decanted supernatant fluid was usually almost water-clear with only a faint opalescence, and for this reason was rapidly filtrable. 1 cc. of a broth culture of *B. prodigiosus* was added to each 15 to 20 cc. of fluid just before it was passed through Seitz or Berkefeld filters. The resulting filtrates were tested for sterility in 1.5 cc. amounts. All filtrates recorded were bacteriologically sterile.

The results of the filtration experiments are summarized in Table I. Warts produced by filtrates, recorded in Table I as positive, were as extensive and characteristic as those in the control animals which had been inoculated with unfiltered suspensions. Furthermore, when domestic rabbits were used as the test animals, filtration, especially through Berkefeld V or N candles, instead of prolonging the incubation period as might be expected because of some possible removal of the filtrable agent by absorption on the filter surface, usually had either no effect or shortened the period. In wild rabbits, from the limited data at hand, it would seem that filtration resulted in a slight prolongation of the incubation period. From the data recorded in Table I it can be concluded that the etiological agent causing warts in rabbits readily passes Berkefeld filters, of V, N, or W porosity but does not regularly pass a Seitz filter when two pads are employed. Filtration through a Seitz filter, using one pad, allowed not only the virus to pass but also *B. prodigiosus*.

No extensive attempts to cultivate visible microbial forms from filtrates of proven infectivity were made. However, during the investigation active filtrates have been cultured repeatedly in plain and blood broth and on plain and blood agar and such cultures have remained sterile both as regards the test organism, *B. prodigiosus*, or any other visible bacterial form. While no special media have been employed in these attempts to demonstrate the bacteriological sterility of active filtrates, the results obtained using the media mentioned above, considered with the fact that sections of actively growing warts or films of

TABLE I

Filtration Experiments

Experiment No.	Filter	Time of filtration	Amount of filtrate	Maximum negative pressure	Rabbit No., inoculated	Result: wart formation on inoculated skin	Incubation period
		min.	*cc.*	*cm. Hg*			*days*
1	Berkefeld V	2	30	62	DR* 621	Positive	6
	Unfiltered suspension				DR 620	"	6
2	Berkefeld V	0.75	30	62	DR 644	"	6
	" N	1	19.5	62	DR 575	"	6
	" "	1	19.5	62	WR† 634	"	9
	" W	2	10.5	62	DR 613	"	8
	" "	2	10.5	62	WR 632	"	9
	Unfiltered suspension				DR 666	"	9
	" "				WR 637	"	7
3	Berkefeld V	0.50	29	62	DR 640	"	7
	Unfiltered suspension				DR 681	"	10
4	Seitz (2 pads)	2	30	Positive pressure	DR 711	Negative	
	" (2 ")	2	30	" "	DR 725	"	
	Berkefeld V	2.5	30	62	DR 729	Positive	12
	" "	2.5	30	62	WR 733	"	12
5	" N	0.5	34	62	DR 790	"	9
	" W	3	27	62	DR 789	"	9
	Unfiltered suspension				DR 791	"	9
6	Seitz (2 pads)	2	34	Positive pressure	DR 793	"	15
	Berkefeld N	1.5	29	62	DR 788	"	7
	" "	1.5	29	62	DR 794	"	7
	" W	3	29	62	DR 784	"	11
	" "	3	29	62	DR 795	"	11
	Unfiltered suspension				DR 792	"	9

* DR = domestic rabbit.

† WR = wild rabbit.

active unfiltered infectious suspensions have failed to reveal the presence of any constant perceptible microbial form, would seem clearly to indicate that no visible organized agent is etiologically essential to the wart production.

Heat Resistance of the Wart-Inducing Agent

The method just described for preparing wart suspensions for filtration was employed in the heating experiments to be outlined. Only the slightly turbid supernatant fluid of centrifuged physiological saline suspensions of glycerolated

TABLE II

Heat Resistance of the Wart-Inducing Agent

Heated for 30 min.	Rabbit No., inoculated	Result: wart formation on inoculated skin	Incubation period
°C.			*days*
45	716, right side	Positive	10
55	715	"	11
60	746, left side	"	7
60	757 " "	"	7
60	713	"	10
60	772	"	8
60	726	"	9
60	785	"	8
65	716, left side	"	10
65	748 " "	"	9
65	759 " "	"	8
65	752 " "	"	8
65	778 " "	"	8
65	738	"	8
65	781, left side	Negative	
67	753 " "	Positive	26 (only 5 warts)
67	779 " "	"	20 (developed well for 10 days, then retrogressed)
67	782, left side	"	20 (developed poorly and retrogressed)
70	753, right side	Negative	
70	778 " "	"	
70	781 " "	"	
73	779 " "	"	
73	782 " "	"	
73	783	"	
75	746, right side	"	
75	757 " "	"	
75	752 " "	"	
75	777	"	
75	780	"	
85	748, right side	"	
85	759 " "	"	

warts was used. The fluid to be heated was sealed in sterile glass ampoules and completely submerged in the water bath for the 30 minute period during which it was exposed to a given temperature.

The results of the heating experiments are given in Table II.

The data recorded in Table II indicate that the activity of the wart-inducing agent is unaffected by temperatures of 65°C. or below for ½ hour but is completely destroyed by temperatures of 70°C. or higher. Heating to 67°C. for 30 minutes, while not completely inactivating the wart-producing agent, did exert a deleterious influence on it. This was evidenced by a marked prolongation of the incubation period in rabbits infected with material heated to this temperature and by scant takes and early retrogression of the resulting warts. It was of interest to note in this respect that the heating of suspensions at temperatures from 45–65°C., instead of lengthening the incubation period in inoculated animals, often shortened it as compared with that shown in animals receiving the unheated control suspensions.

In some of the heating experiments opposite sides of a single rabbit were used to test two suspensions, with adequate care that material from one side did not contaminate the other side. In earlier carefully controlled experiments in which both sides of an animal were shaved and scarified but only one side inoculated with an infectious suspension, it was shown that warts developed only on the inoculated side. In the experiments in Table II, in most instances, inoculations were arranged in such a way that only one side of the animal developed warts.

The thermometer used in these experiments was compared with a standard thermometer and was found to give readings 0.2°C. below those of the standard instrument. This small correction has not been made in the data recorded in Table II.

Routes of Infection

Only the method of inoculation by scarification has yielded constant results in our hands. Inoculation intravenously with infectious Berkefeld filtrates, after first abrading an area of the skin of the abdomen with a sterile needle, led to infection of the abraded areas in two out of four cases. Of the two positive animals, one, a wild rabbit, developed only a single wart; while the other, a domestic rabbit, developed four warts on the abraded area and two on the back of the neck. The incubation period in both of these cases was over three times as long as that of the control animals infected by scarification. At autopsy, all four intravenously inoculated animals were free from visceral pathology ascribable to the wart-inducing agent. Inoculations of either wild or domestic rabbits intraperitoneally, subcutaneously, intratesticularly, or intracerebrally, with filtrates of proven infectivity on scarification, have yielded entirely negative clinical and pathological results. About 50 per cent of the intradermal inoculations resulted in wart formation although in these instances the warts appeared not at the point where the inoculum had been deposited but at the point where the needle had pierced the epidermis and where some of the inoculum had leaked from the needle tract. The incubation period of warts produced in this way was always longer than when infection had been accomplished by scarification.

Resistance of Infected Rabbits to Reinfection

In a series of 123 wild and domestic rabbits inoculated with suspensions of known infectivity, we have encountered no animal that was naturally immune.

One of the two rabbits in which warts underwent complete retrogression was tested and found to be resistant to reinfection. The serum of this animal, however, when mixed in equal parts with an infectious suspension, failed to neutralize the wart-inducing agent. It did prolong the incubation period considerably.

Ten domestic rabbits carrying warts of various ages have been tested for immunity to reinfection. Five resisted reinfection successfully, while the remaining five, after unusually long incubation periods, finally developed warts at the sites of their new inoculations. These warts were much less numerous and slower in growth than those in the control animals. The time elapsing between the primary infection and the attempt at reinfection was apparently of little importance, for two animals were found to be susceptible to reinfection 76 days after their primary inoculation while one animal was completely resistant to reinfection 31 days after its primary inoculation. Two rabbits tested 14 days after their primary inoculation and 6 days after the first appearance of warts possessed some resistance which was evidenced by the fact that no warts appeared at the sites of their new inoculations for

24 days, whereas the incubation period in the control animals was 8 days.

Three wild rabbits that were carrying warts when received from Kansas were tested for immunity. All three were still susceptible, although the incubation periods were markedly lengthened. One experimentally infected wild rabbit has been found to be immune to reinfection.

Neutralizing Properties of the Sera of Infected Rabbits

Sera from wild rabbits, either naturally or experimentally infected, as well as the sera from experimentally infected domestic rabbits, have been found to contain antibodies effective against the wart-inducing agent. Most of such sera have neutralized it completely, so that warts failed to develop in animals inoculated with mixtures of serum and the infectious agent; in the others, partial neutralization was evidenced by a doubling or trebling of the incubation period.

For these tests the usual virus neutralization technique was employed. Equal parts of serum and infectious suspension were mixed and stored overnight (17 hours) in the refrigerator. The control consisted of equal parts of infectious suspension and normal rabbit serum. Rabbits were inoculated with these mixtures on the freshly scarified skin in the usual way, using one shaven side for the control inoculation and the other for the neutralization test, performing both inoculations on the same rabbit and thus avoiding possible individual variations in resistance.

From the above experiments it is evident that an active wart infection in rabbits not only renders them completely or partially resistant to reinfection but also that it evokes antibodies, demonstrable in their sera, capable of completely or partially neutralizing the wart-inducing agent.

Wart-Producing Agent Not Immunologically Related to the Viruses Causing Infectious Fibroma or Myxoma

In earlier experiments (1), a benign fibroma-like new growth of rabbits caused by a filtrable virus was found capable of establishing a resistance in rabbits to fatal infection with the otherwise uniformly deadly virus of infectious myxoma. To explore the possibility of an immunological relationship between the wart-producing agent and the benign fibroma virus or the fatal myxoma virus, a number of experiments were conducted. It was found that rabbits infected with the wart-producing agent and carrying large warts at the time of testing were fully susceptible to both the fibroma and the myxoma viruses. No alteration of the normal course of either of these diseases was observed as the result of previous infection with the wart-producing agent. Conversely, rabbits recovered from infection with fibroma virus and demonstrably immune to reinoculation with that virus were still fully susceptible to infection with the wart-producing agent. Rabbits immunized against infectious myxoma by preliminary infection with fibroma virus and subsequent inoculation with *Virus myxomatosum*, and possessing demonstrable virucidal antibodies for *Virus myxomatosum*, were also still fully susceptible to infection with the wart-producing agent. These experiments indicate that the wart-producing agent is not immunologically related to either of these viruses.

It may be noted here that in the original glycerolated wart material obtained from Iowa both the wart-producing agent and the virus of infectious fibroma were present. The latter was easily separated from the former by testicular passage through domestic rabbits. The strain of fibroma virus thus isolated was typical in all major respects of the original strain described earlier (2), and like it was capable of protecting rabbits against fatal infection with *Virus myxomatosum*.

Attempts to Transmit the Wart-Producing Agent in Series through Rabbits

In all, twenty-six domestic and wild rabbits have been inoculated in the usual way with suspensions prepared from experimentally engendered domestic rabbit warts ranging in age from 1 to 116 days. Not only did all such inoculations yield negative results but the animals, when subsequently tested, were found to be still fully susceptible to infection with the wart-producing agent from wild rabbit papillomata. On the other hand, either naturally occurring or experimentally produced warts from wild rabbits proved readily transmissible to either wild or domestic rabbits. Warts from nine naturally occurring cases of the disease in wild rabbits have been tested and all found to be infectious for both wild and domestic rabbits. In like manner, experimentally produced warts from nine wild rabbits have been tested for infectivity. Eight of these proved infectious for either domestic or wild rabbits while the warts from one proved to be non-transmissible.

We have not yet attempted to pass the wart-producing agent through a long series of wild rabbits but in the course of obtaining fresh infectious material it has at present reached its third serial passage. In spite of the fact that the agent cannot be propagated in series through domestic rabbits, it is probable that it can be passed indefinitely in series through wild rabbits and that any of these serial wild rabbit passages can be used in infecting domestic rabbits.

No attempt has so far been made to transmit the domestic rabbit warts by means of tissue grafts, although in a small number of experiments freshly prepared cell-containing suspensions of young actively growing papillomata from domestic rabbits have yielded negative results when inoculated intracutaneously or subcutaneously into domestic rabbits. Instead, it has seemed best to study the rabbit papillomata first as an infectious process caused by a filtrable agent and to determine, if possible, why this agent should be readily transmissible in series when inducing warts in wild rabbits and non-transmissible when inducing similar growths in domestic rabbits.

That the degree of maturity of the warts in domestic rabbits at the time that attempts were made to transmit them in series was not a determining factor is indicated by the fact that warts taken at intervals of 6 to 8 days, from their first appearance until they were 116 days old, yielded no successful infections.

Domestic rabbit warts glycerolated for varying periods of time were repeatedly tested for infectivity to determine whether or not glycerol storage has an activating effect on the agent as it does on herpes virus of low activity (3–5). The results of these experiments were all negative.

In a series of experiments conducted before the presence of neutralizing antibodies in the blood serum of wart-bearing animals had been demonstrated, it was found that when an inactive domestic rabbit wart suspension was mixed with an equal part of a suspension prepared from wild rabbit warts of known infectivity, the resulting mixture was either completely non-infectious or the incubation period was prolonged and the resulting warts few in number as compared with control animals. This suggested the presence in warts from domestic rabbits of an inhibitory substance similar to that found by Sittenfield, Johnson, and Jobling (6) and Murphy, Helmer, Claude, and Sturm (7) in fowl tumors. In the light of subsequent experiments in which the sera of wart-bearing rabbits were found to neutralize partially or completely the wart-producing agent, it seems possible that the inhibitory properties observed in non-infectious domestic rabbit wart suspensions might in reality have been due to contained humoral antibodies. A point of argument against this belief is that, while humoral antibodies were demonstrable in the sera from both infected wild and domestic rabbits, only the domestic rabbit warts possessed demonstrable inhibitory properties. We have as yet made no systematic attempt to render experimental domestic rabbit warts infectious by removal of a hypothetical inhibitory substance. We have tried, however, to infect rabbits with inactive experimental domestic rabbit wart suspensions that had been heated to 60°C. for 30 minutes in the hope that that temperature might inactivate the possible inhibitor without affecting the wart-producing agent, with suspensions prepared from domestic rabbit wart cells that had been washed repeatedly and sufficiently to remove all freely soluble humoral antibody, and with Berkefeld filtrates of inactive wart suspensions. All three of these procedures yielded completely negative results. Both the Iowa and the Kansas strain of the disease were used in these attempts to transmit warts in series through domestic rabbits.

DISCUSSION

The absence of significant visible bacterial forms in highly active wart-producing suspensions together with the ready filtrability of the etiological agent and the inability to cultivate, on lifeless media, any visible microbial form from demonstrably active filtrates; the agent's ability to transmit in series through wild rabbits; its glycerol resistance; its ability to induce in its hosts an immunity which is constant although of variable degree; and its apparent tropism for one type of tissue place this agent in the filtrable virus group.

The non-transmissibility of the agent in series through one of its demonstrably susceptible hosts, the domestic rabbit, is not a characteristic of most of the known virus diseases. An analogy, however, is to be found in the group of filtrable fowl tumors. Des Ligneris (8), working with Rous Sarcoma 1 of chickens, has found that while both turkeys and guinea fowls are susceptible, transmission through these two alien species is limited to two successive serial passages. Similarly, Andrewes (9) has found that while Rous Sarcoma 1 will produce fatal metastasizing tumors in its first pheasant passage it cannot be transmitted in its characteristic form from pheasant to pheasant.[2] It seems probable that the domestic rabbit (genus *Oryctolagus*) is sufficiently distantly related to the wild cottontail rabbit (genus *Sylvilagus*) to behave towards infection with a filtrable new growth of wild rabbit origin in much the same manner as do turkeys, guinea fowls, and pheasants towards infection with a filtrable chicken tumor.

Another property of the wart-producing agent that is unusual among viruses causing diseases in animals is its resistance to heat. Suspended in 0.9 per cent NaCl solution it proved capable of withstanding a temperature of 65°C. for 30 minutes in sealed ampoules without apparent damage to its wart-producing properties. Virus heated to 67°C. for 30 minutes, while still active, produced, in our limited number of experiments, warts which either developed scantily or retrogressed after a few days' growth. We are aware of no other animal virus which will withstand so high a temperature in the moist state; most are completely inactivated at much lower temperatures. However, among the plant viruses, which are on the whole as susceptible as animal viruses to the effects of heat, there are several which withstand heating to 65°C. or more (10). The virus of tobacco mosaic is an example of a typical plant virus that is relatively heat resistant (11). For this reason it does not seem necessary to consider seriously the possibility that the unusual heat resistance of the wart-producing agent eliminates it from classification as a virus.

The not infrequent shortening of the incubation period in animals inoculated either with virus heated to from 45–65°C. or with virus that had been filtered through Berkefeld V or N candles cannot be explained. Removal of an inhibiting agent by these two procedures is suggested by the data.

In the gross and histologically, the warts of rabbits described in this paper are typical of virus-produced papillomata (12–15) as known in man, cattle, and dogs. It has not been previously observed in studies of mammalian warts of this kind that an epithelial neoplastic process of identical gross and histological appearance can be induced in two ani-

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[2] Andrewes has recently reported the successful serial passage of Rous Sarcoma 1 through pheasants (Andrewes, C. H., *J. Path. and Bact.*, 1933, 37, 17).

mal species, in one of which the condition is not only transmissible in series, but transmissible by cell-free filtrates, and in the other of which it is not transmissible at all. Here then in what is certainly a single clinical entity are examples of the two extremes of neoplastic processes considered from the standpoint of transmissibility. In the wild rabbit the papillomata can be initiated by inoculating the animal with a filtrable agent and they are transmissible in series by inoculation with filtered or unfiltered virus. From an etiological standpoint, then, the wild rabbit warts are analogous to the chicken tumors which by some are not considered as true representatives of neoplastic processes simply because they are transmissible by cell-free filtrates. Thus the wild rabbit papilloma represents the one extreme of a tumor induced by an infectious agent which can be separated from the cells and some of whose properties can be studied.

The other extreme is exemplified by the papillomata induced in domestic rabbits which, while initiated by the same virus, have so far resisted transmission either to domestic or wild rabbits. These are thus analogous to many of the tumors of mammals which cannot be transmitted in series by the usual methods of transplantation. No objection to the eligibility of the domestic rabbit warts for consideration as neoplastic processes could be raised on the grounds that a causative agent distinct from the proliferating cells can be discriminated. A study of this epithelial new growth in domestic rabbits without knowledge of its causation would probably lead an investigator to classify it as one of that large group of so called "spontaneous" mammalian tumors that are non-transmissible. It would not even be suspected that the papillomata had been caused by a filtrable virus of wild rabbit origin.

The question which is naturally brought to mind by the experiments of des Ligneris (8) and Andrewes (9) with fowl tumors and our own with rabbit warts is whether certain "spontaneous" non-transmissible or not readily transmissible tumors may not originally have been caused by viruses which produce transmissible tumors in some other species. A careful study, from this point of view, of the causes underlying the non-transmissibility of these various tumors may bring to light new knowledge of the etiology of neoplastic processes in general, especially in the group of mammalian tumors which are either entirely non-transmissible or transmissible only by viable cell-containing grafts.

SUMMARY

A papilloma has been observed in wild cottontail rabbits and has been found to be transmissible to both wild and domestic rabbits. The clinical and pathological pictures of the condition have been described. It has been found that the causative agent is readily filtrable through Berkefeld but not regularly through Seitz filters, that it stores well in glycerol, that it is still active after heating to 67°C. for 30 minutes, but not after heating to 70°C., and that it exhibits a marked tropism for cutaneous epithelium. The activities and properties of the papilloma-producing agent warrant its classification as a filtrable virus.

Rabbits carrying experimentally produced papillomata are partially or completely immune to reinfection and, furthermore, their sera partially or completely neutralize the causative virus. The disease is transmissible in series through wild rabbits and virus of wild rabbit origin is readily transmissible to domestic rabbits, producing in this species papillomata identical in appearance with those found in wild rabbits. However, the condition is not transmissible in series through domestic rabbits. The possible significance of this observation has been discussed. The virus of infectious papillomatosis is not related immunologically to either the virus of infectious fibroma or to that of infectious myxoma of rabbits.

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EXPLANATION OF PLATES

PLATE 33

FIG. 1. Spontaneous wart on the thigh of a wild rabbit. The vertically striated appearance of the lateral surfaces can be seen. The upper portion of the growth is hard and well keratinized.

FIG. 2. Experimental warts on the abdomen of a domestic rabbit showing individual discrete wart formation in an animal infected following scarification by needle. These warts are 23 days old.

FIG. 3. Same animal as shown in Fig. 2. The warts are now 52 days old and, considered individually, are accurate reproductions of the spontaneous warts seen in wild rabbits.

FIG. 4. Experimental warts on the abdomen of a domestic rabbit showing massive confluent wart formation in an animal infected following scarification with sandpaper. These warts are 118 days old and are firmly keratinized.

PLATE 34

FIG. 5. Section of a spontaneous wart in a wild rabbit. The long, branching papillae are capped by an enormous amount of keratinized material. Iron alum hematoxylin and eosin. × 10.4.

FIG. 6. Higher power of a spontaneous wart showing the tall and narrow germinal cells, the great thickness of the polygonal cell and granular layers, and the imperfectly keratinized surface layer. Iron alum hematoxylin and eosin. × 168.

PLATE 35

FIG. 7. Section of an experimental wart in a domestic rabbit 3 days after its appearance. The mass of proliferating epithelium lies partly below the level of the normal epithelium, which it underlies at the margins. As yet there is no excess keratinization. Iron alum hematoxylin and eosin. × 33.

FIG. 8. Section of an experimental wart in a domestic rabbit 18 days after its appearance. The epithelium is greatly thickened and the new growth projects considerably from the surface. Iron alum hematoxylin and eosin. × 77.

FIG. 9. Still later stage of the experimental disease in a domestic rabbit (36 days). The features of the spontaneous growth are by now fairly faithfully reproduced. Iron alum hematoxylin and eosin. × 26.

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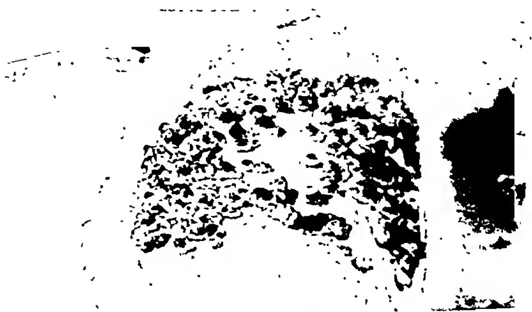

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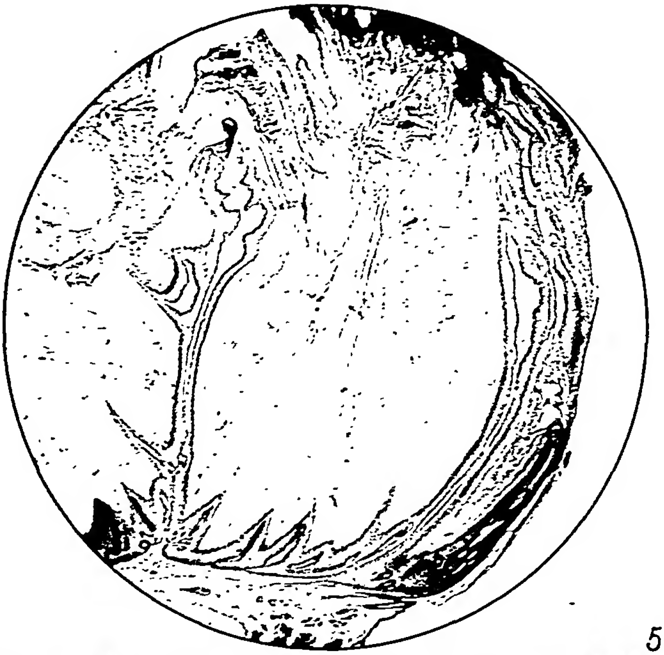

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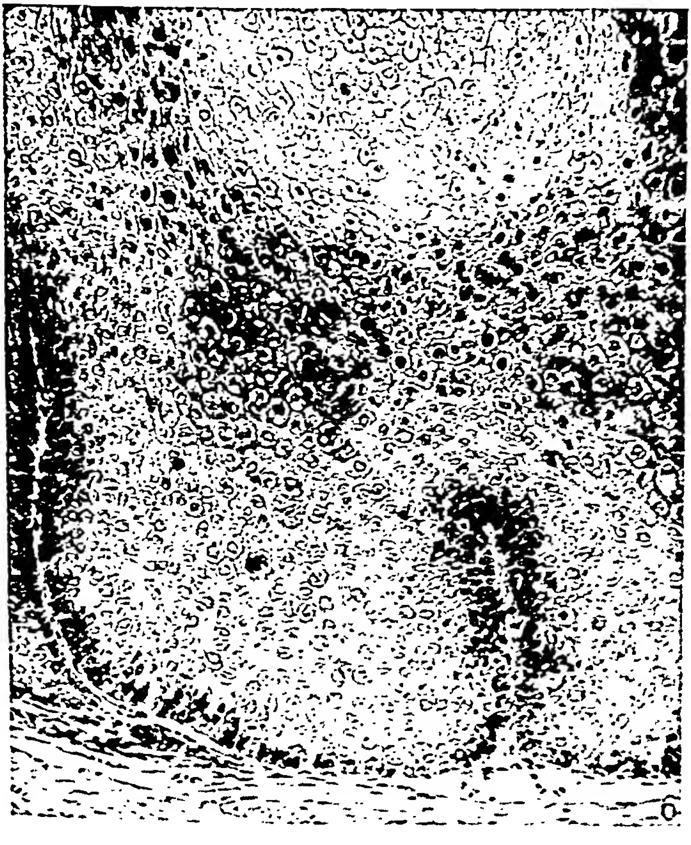
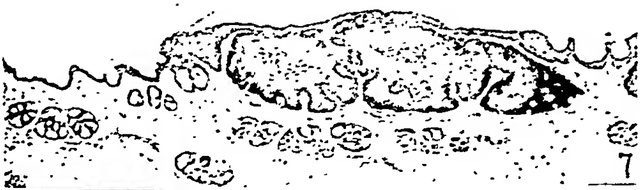


(Shope: Infectious papillomatosis of rabbits)



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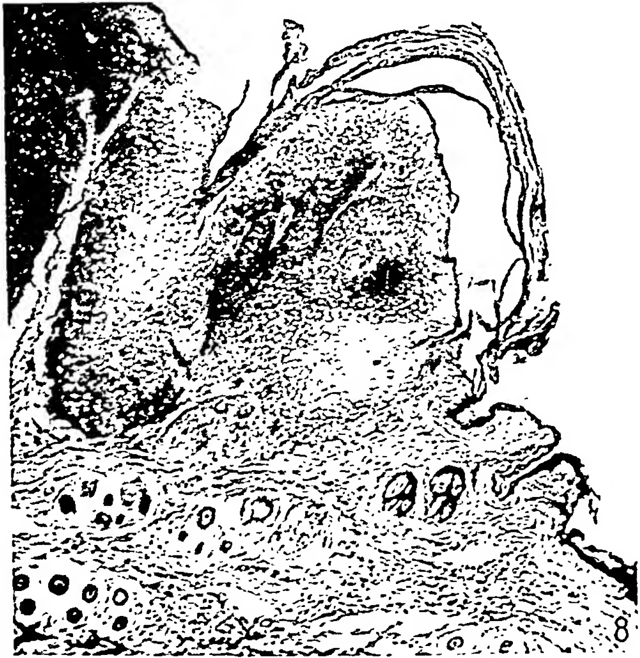

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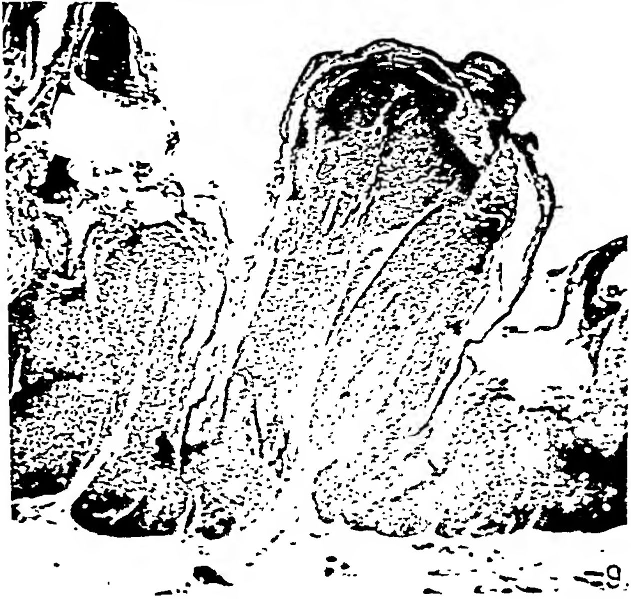

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AN IMMUNOLOGICAL STUDY OF NATIVE, DENATURED, AND REVERSED SERUM ALBUMIN*

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The denaturation of proteins has long been considered an irreversible process. This view is still generally prevalent in spite of evidence which has accumulated showing that denatured protein may, under appropriate conditions, be reversed into soluble material which exhibits the same physical and chemical properties as the original native protein. The transformation of denatured hemoglobin and globin to their native states has been extensively studied by Anson and Mirsky (1). In no case could they detect any physical or chemical differences between their original substance and the one obtained by the reversal of the thoroughly denatured protein. In 1931 they extended their observations to serum albumin (2) and showed that it was possible to prepare a soluble, heat-coagulable, crystalline protein from albumin which had been denatured by heat, acetone, or trichloracetic acid. The reversal of heat-denatured serum albumin from acid solution had been observed as early as 1910 by Michaelis and Rona (3). These investigators did not crystallize the soluble material recovered from the denatured protein, nor did they attempt to apply any other criteria of reversal. Recently Northrop has reported the reversal of denatured pepsin (4) and trypsin (5). The reversal of denatured egg albumin has not been accomplished by the present methods.

The technique involved in the reversal process is simple, especially with serum albumin. Anson and Mirsky (2) dissolved denatured serum albumin, prepared by the action of heat, acetone, or trichlorace-

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* This investigation was carried out during tenure of the John Ware Memorial Student Fellowship, 1931–33, and presented as a thesis for the degree in Medicine at the Harvard Medical School.

tic acid, in an acid solution. When this solution was neutralized with sodium hydroxide at room temperature, it yielded about 65 per cent soluble protein at the isoelectric point. All this soluble protein could be crystallized into a form resembling native albumin.

In view of the fundamental importance of the reversal process in understanding the mechanism of denaturation it appeared desirable to apply another criterion of reversibility. This we felt could be accomplished better by immunological, than by any but the most exhaustive chemical and physical methods at present available for protein characterization.

A study of the reversal of heat-denatured horse serum albumin by means of the precipitin reaction was therefore undertaken. It had been shown by Obermayer and Pick (6) that heated serum proteins lose most of their ability to react with precipitin antibodies obtained by the injection of the native protein. Later Schmidt (7) obtained substantially the same results. It thus appeared feasible to employ this method to determine the relationship of the reversed albumin to ts native and denatured precursors.

Experimental Methods

Native, heat-denatured, and reversed heat-denatured horse serum albumins were prepared by the technique of Anson and Mirsky (2) modified, in certain respects, for an immunological study. Amorphous serum albumin was chosen, as it had been studied by Anson and Mirsky. Moreover, Svedberg (8) has shown that repeated crystallization of serum albumin gives an increasingly heterogeneous mixture; and it has been known to immunologists that the crystalline albumin is a poorer antigen than the amorphous form.

The specific sera were obtained by sensitizing rabbits to each of the three antigens. These sera were tested against the three antigens by means of the ring test precipitin method. The antigen dilutions were made on a comparable basis by expressing weight of total protein per volume of saline. A detailed description of the antigen preparations and the immunological procedures is given below.

Native Serum Albumin.—Prepared by half saturating horse serum with $(NH_4)_2SO_4$ and discarding the globulin precipitate. Solid $(NH_4)_2SO_4$ was then added to the filtrate (200 gm. per liter) for precipitation of the albumin fraction. This amorphous albumin was dialyzed against cold distilled water in cellophane membranes for 24 to 48 hours until practically salt-free. A 2 per cent solution was used for sensitization of animals and for dilutions in the precipitin tests, as well as for the preparations below.

Heat-Denatured Albumin.—Prepared as follows: 100 cc. of 2 per cent native albumin were acidified with 30. cc. of N/10 HCl. The solution was stirred in a bath of boiling water until it reached 90°C. The heating was then continued for exactly 3 minutes, during which time the temperature usually rose to 97°C. The hot solution was neutralized with sufficient N/10 NaOH to give maximum coagulation (about 22.5 cc.) and then cooled at once to room temperature. Upon neutralization, a heavy precipitate of coagulated albumin appeared. It was separated by centrifugation, suspended and thoroughly stirred in 100 cc. of 0.9 per cent NaCl, and again centrifuged. This washing was repeated 5 times with 100 cc. portions of 0.9 per cent saline in order to remove any undenatured serum protein which might have been present.

The coagulated albumin dissolved incompletely at pH 7 by addition of NaOH at room temperature, but it was readily rendered soluble by suspending the denatured albumin in 75 cc. of distilled water, warming to 90°C., and dissolving with sufficient N/10 NaOH to give a clear solution. It was then immediately neutralized to pH 7 with the required amount of N/10 HCl, and cooled at once to room temperature by immersion in an ice bath. The amount of HCl needed was previously determined by means of an aliquot. This procedure did not subject the denatured albumin to such conditions of pH or temperature as would favor reversal; and resulted in a slightly opalescent solution which was analyzed for total protein, and was satisfactory for injections and for the precipitin tests. Although there was an additional manipulation involved in this process, it was deemed permissible since the rate of heat denaturation at reactions slightly alkaline to the isoelectric point is negligible, in comparison with the rate in acid solution (9).

Reversed Heated Albumin.—Prepared from 100 cc. of 2 per cent native albumin which had been heated, coagulated, and washed as described above. The denatured protein precipitate was dissolved in 15 cc. of N/5 HCl and diluted to 60 cc. This clear solution of denatured albumin was then reversed at room temperature by addition of sufficient N/5 NaOH to bring the pH to 4.7 (determined by methyl red as indicator). The reversed solution was only slightly turbid at this pH, indicating that most of the denatured albumin had regained solubility at the isoelectric point. Half saturation with $(NH_4)_2SO_4$ resulted in considerable precipitation. This procedure is recommended by Anson and Mirsky to remove any denatured albumin held in solution. It by no means follows that all of the precipitated protein is denatured, since even the solubility of native serum albumin in half saturated $(NH_4)_2SO_4$ near the isoelectric pH is distinctly limited. The filtrate from the half saturated solution containing the soluble, reversed albumin was dialyzed in a cellophane membrane against cold distilled water until practically salt-free. It was then concentrated in the cold by means of a Sørensen type of negative pressure dialyzer (10) to approximately 1 per cent. After analysis for total protein this solution was used for the injection and immunological procedures. For convenience in the tables, it has been designated as reversed heated albumin.

Preparation of Antisera.—Three rabbits were injected with each of the above albumins both intravenously and subcutaneously until they gave a precipitating titer of at least 1:100,000. Eight of the nine animals showed a satisfactory response. Usually three injections of 5 to 20 mg. of total protein were given each week for 3 to 5 weeks.

Precipitin Tests.—The three antigens were diluted with 0.9 per cent saline. The dilutions are on a quantitative basis, all being reducible to grams of total protein (Kjeldahl nitrogen multiplied by 6.25) per cubic centimeter of saline. Thus a 1:100,000 dilution of antigen corresponds to 1 gm. of albumin in 100,000 cc. of saline, and is not directly comparable with the total protein in dilutions of whole serum unless one corrects for the original approximately 1:12 dilution of protein in horse serum.

The tests were made by layering about 0.3 cc. of the antigen dilutions over an equal volume of the antisera and reading the ring which formed at the interface. The following notation was used throughout in order to put the tests on a semi-quantitative basis.

++++ = heavy ring which formed within 2 minutes.
+++ = heavy ring at 20 minutes.
++ = moderate ring at 20 minutes.
+ = faint ring at 20 minutes.

Thus readings were taken at 2 minutes and the 4 plus rings recorded. The remainder were read at the end of 20 minutes. The unknowns were always compared with control solution of (1) saline + specific antisera, and (2) 1:1,000 dilutions of the antigens + normal rabbit serum.

RESULTS

The reactions of the native, heat-denatured, and reversed heated albumins with sera prepared by injection of native albumin are shown in Table I. The results show a marked decrease in the reactivity of the heat-denatured albumin toward the native precipitin, confirming the results of Obermayer and Pick (6) and of Schmidt (7). The table also indicates a striking increase in the precipitability of the reversed heated albumin by native precipitin as compared with the denatured antigen. The reactivity is approximately equal to that of native serum albumin since both react in dilutions of 1:100,000 which, as previously explained, contain the same weight of total protein for both antigens.

The reactions of the reversed protein with native precipitin were not considered sufficient evidence for this study because it might be argued

TABLE I

Precipitin Reactions with Anti-Native Albumin Sera

Rabbit No.	Antigen	Antigen dilutions							
		1:1,000	1:10,000	1:25,000	1:50,000	1:75,000	1:100,000	1:200,000	1:300,000
78	Native serum albumin	++++	++++	++++	++	++	+	–	–
	Heat-denatured albumin	++	+	–	–	–	–	–	–
	Reversed heated albumin	++++	++++	+++	++	+	+	–	–
79	Native serum albumin	++++	++++	++++	++	++	+	–	–
	Heat-denatured albumin	+	+	+	–	–	–	–	–
	Reversed heated albumin	++++	++++	++++	++	++	+	–	–

Controls: 0.9 per cent saline + Sera 78 and 79, negative.
1:1,000 dilutions of the antigens + normal rabbit serum, negative.

TABLE II

Precipitin Reactions with Anti-Heat-Denatured Albumin Sera

Rabbit No.	Antigen	Antigen dilutions							
		1:1,000	1:10,000	1:25,000	1:50,000	1:75,000	1:100,000	1:200,000	1:300,000
83	Native serum albumin	++++	++++	++	++	++	+	–	–
	Heat-denatured albumin	++++	++++	++	++	++	+	–	–
	Reversed heated albumin	++++	++++	++	+	+	+	–	–
84	Native serum albumin	++++	++	++	+	+	+	–	–
	Heat-denatured albumin	++++	++	++	+	+	+	–	–
	Reversed heated albumin	++++	++	++	+	+	+	–	–
85	Native serum albumin	++++	+++	+++	++	+	+	–	–
	Heat-denatured albumin	++++	++++	++++	++	++	+	–	–
	Reversed heated albumin	++++	++++	++++	++	++	+	–	–

Controls: 0.9 per cent saline + Sera 83, 84, and 85, negative.
1:1,000 dilutions of the three antigens *vs.* normal rabbit serum, negative.

that the reversed protein was acting as a partial antigen. To guard against this possibility we obtained additional results which show the reactions of the various antigens with sera obtained by injecting both denatured and reversed protein. The fact that the reversed protein gave rise to antibodies is evidence that it is a complete and not a partial antigen.

First let us consider the reactions of the three antigens with sera obtained by the injection of heat-denatured albumin, as set forth in Table II. They show an equal reactivity of all three antigens with the specific sera. The phenomenon that heat-denatured serum proteins produced an antibody which reacted equally well with both native and heat-denatured serum was observed by Obermayer and Pick (6). This type of antibody is now well known in immunology by the name coctoprecipitin. Our chief interest in the coctoprecipitin was whether or not the reversed heated antigen would give rise to this peculiar antibody.

TABLE III

Precipitin Reaction with Anti-Reversed Heated Albumin Sera

Rabbit No.	Antigens	Antigen dilutions							
		1:1,000	1:10,000	1:25,000	1:50,000	1:75,000	1:100,000	1:200,000	1:300,000
88	Native serum albumin	++++	++++	++++	+++	+++	++	+	−
	Heat-denatured albumin	+++	+	+	−	−	−	−	−
	Reversed heated albumin	++++	++++	++++	+++	+++	++	+	−
89	Native serum albumin	++++	++++	+++	+++	+	+	−	−
	Heat-denatured albumin	+++	++	+	−	−	−	−	−
	Reversed heated albumin	++++	++++	++++	+++	+	+	−	−
90	Native serum albumin	++++	++++	++++	++++	++	++	+	−
	Heat-denatured albumin	++++	+++	++	+	−	−	−	−
	Reversed heated albumin	++++	++++	++++	++++	++	++	+	−

Controls: 0.9 per cent saline + Sera 88, 89, and 90, negative.
1:1,000 dilutions of the three antigens *vs.* normal rabbit serum, negative.

The reactions of the same three antigens with specific sera obtained by injecting reversed albumin are given in Table III. A comparison of Tables I and III shows that the reversed heated albumin did not act like the denatured albumin and give rise to a coctoprecipitin; but produced an antibody indistinguishable from the native precipitin.

A considerable interaction of the heat-denatured albumin with the native and reversed antisera is indicated at the lower dilutions. This apparently is a partial function of the severity of the denaturation process, for, as shown by Schmidt (7), the cross-reaction varies inversely with the temperature and duration of the heating. Also, sera with high titers against native protein show more reaction with heated antigen than sera with low titers (6). Our results were all obtained with sera of high titer.

We accordingly wished to study the immunological effect of the reversal process on native albumin which had been treated more drastically than the heat-denatured protein. We therefore denatured serum albumin by exposure to acidified acetone for 24 hours, and reversed it in the usual manner as detailed below.

Acetone-Denatured Albumin.—Prepared after Anson and Mirsky (2) with certain departures necessitated by our special needs. 70 cc. of 4.5 per cent native horse albumin (prepared as before) were acidified to 0.05 normal by the addition of 5 N HCl. The albumin was precipitated by the addition of 700 cc. of acetone containing 1.4 cc. of 5 N HCl. The mixture was allowed to stand at room temperature for 24 hours with occasional shaking. The coagulated albumin was then filtered off and dried between filter papers for 2 hours. After pulverization, it was dissolved in 150 cc. of distilled water. The denatured albumin hydrochloride went into solution readily giving a pH of 3.5 to 4. It was a solution prepared by this method that Anson and Mirsky neutralized at room temperature to obtain reversal of acetone-denatured albumin. Since we wished to obtain a denatured albumin at pH 7.0 for comparison with the reversed acetone-denatured albumin, we found it necessary to approach the isoelectric point at a high temperature where reversal could not take place. This was accomplished by warming the acid solution of denatured albumin to 90°C. and neutralizing with N/10 NaOH until all the protein precipitated out. This precipitate was then washed 6 times with 100 cc. portions of 0.9 per cent NaCl. A portion was brought into solution by warming to 90°C. and bringing to pH 7.0 exactly as described before with the heat-denatured albumin. The protein is referred to as acetone-denatured albumin for convenience in the text; but it should be borne in mind that it had been exposed to dilute acid and heat as well as excess of acetone. Hence it was much more violently denatured than the heat-denatured albumin described above.

Reversed Acetone Albumin.—Prepared from the remainder of the washed precipitate of coagulated albumin. This was dissolved in N/5 HCl and reversed and dialyzed exactly as described above for the reversed heated antigen (page This protein is designated reversed acetone albumin in the tables.

Results with Acetone-Denatured and Reversed Acetone Albumins

These two antigens were tested against one of the sera obtained injecting native albumin. The technique and nomenclature of precipitin reactions are the same as previously used except that readings were taken at the end of 90 minutes. The results are s forth in Table IV where, even more strikingly than before, the contras between the reactivity of the denatured albumin and its reverse derivative is demonstrated. This drastically treated denature albumin showed no reactivity with native precipitin even in the lowest dilution, whereas the reversed material prepared from it reacted in a dilution of 1:200,000 exactly as did the same weight of native albumin.

TABLE IV

Precipitin Reactions with Anti-Native Albumin Serum

Rabbit No.	Antigen	Antigen dilutions 1:1,000	1:10,000	1:25,000	1:50,000	1:75,000	1:100,000	1:200,000	1:300,000
78	Native serum albumin	+++	+++	+++	++	++	+	+	−
	Acetone-denatured albumin	−	−	−	−	−	−	−	−
	Reversed acetone albumin	+++	+++	++	++	++	+	+	−

Controls: 0.9 per cent saline + Serum 79, negative.
1:1,000 dilutions of antigens *vs.* normal rabbit serum, negative.

Because of the general agreement of the data obtained, we did not sensitize animals to the acetone-denatured and reversed acetone albumins and study their interactions.

DISCUSSION

These data indicate that, as far as can be shown by the precipitin reaction, there is no difference between native and reversed serum albumin. This is not proof, of course, that the two proteins are identical, but is evidence that they do not differ in the portions of the molecule which exert the specific influence in the precipitin reaction.

It seems reasonable when one considers the delicacy of the biological method employed to suppose that, if the reversed protein is not identical in all parts of its complex molecule with the native albumin, it is closer, at least, to the native than the denatured state. This is suggested by the failure to form a coctoprecipitin by the reversed albumin and its production of a precipitin indistinguishable from that produced by the native albumin.

There are also possible applications of these results in immunology. It is apparent how carefully any antigen such as horse serum must be described in terms of hydrogen ion activity, for it would be obviously useless to compare a native with a denatured serum if the latter had been exposed to such conditions of pH as to result in reversal of the denaturation. Furthermore, the marked changes in antigenic properties produced by the chemical manipulations involved in the reversal process may give some insight into the nature of immunological specificity, especially when more exact chemical and physical characterizations of native and denatured proteins become available.

The author is indebted to Dr. Edwin J. Cohn for helpful suggestions during the course of this research.

CONCLUSION

Native and reversed horse serum albumin are indistinguishable when tested immunologically by means of the precipitin reaction.

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FURTHER OBSERVATIONS ON THE CULTIVATION OF VACCINE VIRUS FOR JENNERIAN PROPHYLAXIS IN MAN

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In 1913, Steinhardt, Israeli, and Lambert (1) demonstrated that vaccine virus is capable of multiplication in the presence of bits of viable tissue embedded in plasma. Although this work has been confirmed by a number of investigators, cultures of vaccine virus made by means of the cover-slip technique have proved of no value in the preparation of an active agent for Jennerian prophylaxis. In 1927, Carrel and Rivers (2) devised a method for the cultivation of vaccine virus in which bits of viable chick embryo tissue embedded in plasma in flasks were used. In 1928, Maitland and Maitland (3) showed that they were able to grow vaccine virus in a medium consisting of minced hen kidney suspended in a mixture of hen serum and Tyrode's solution. In 1929, Rivers, Haagen, and Muckenfuss (4) demonstrated that cells remain viable for at least 5 days in a medium made according to Maitland's directions. However, if the cells are killed by repeated freezing and thawing, the medium no longer supports the multiplication of virus.

In a further search for a simple and safe method of cultivating vaccine virus for human use, we devised a highly satisfactory medium which consists of bits of minced chick embryo tissue (0.1 gm.) suspended in Tyrode's solution (4–5 cc.) in "collar flasks." In 1930, the results obtained with cultures of a neurovaccine virus were reported (5). In 1931, we described (6) our work with a dermal strain of vaccine virus and reported that the culture virus had been successfully used for the vaccination of 3 children. In 1932, Herzberg (7) reported that he had been able to vaccinate human beings with virus cultivated in the manner described by us. Since our last report in 1931 we have continued our observations, and it seems appropriate at this time to record certain facts that have accumulated in regard to the culture virus and its use for Jennerian prophylaxis.

One of the objects of our work on the cultivation of vaccine virus has been to obtain an active agent, free from bacteria and contaminating viruses, that will protect human beings against smallpox with the least amount of inconvenience and discomfort to the individuals vaccinated. Consequently, all cultures of virus used in this type of work have been carefully tested for the presence of bacteria and other undesirable agents.

EXPERIMENTAL

As we have been able to show (5, 6) that vaccine virus will multiply in the presence of bits of minced chick embryo tissue (0.1 gm.) and Tyrode's solution (4–5 cc.), we decided to determine what would happen to the active agent when it was cultivated in such a medium over a long period of time. This was accomplished by making transfers in series from old cultures to flasks of fresh medium at intervals of 4 or 5 days and then testing the activity of the virus in the different culture passages by means of dermal and intradermal titrations in rabbits and vaccination of human beings.

Effect of in Vitro Cultivation on the Activity of Vaccine Virus in the Rabbit

Cultures of vaccine virus were initiated, March 9, 1931, and the titer in rabbits of the active agent in the 1st set of cultures was 10^{-6}. The titer of the virus in the 19th set of cultures was 10^{-6}, in the 30th 10^{-4}, in the 60th 10^{-2}, in the 80th 10^{-2}, in the 86th 10^{-1}. From the 88th set of cultures to the 99th only the undiluted virus produced a lesion when injected intradermally. From the record portrayed in Chart 1 it can be seen that the titer of the virus in the cultures maintained a high level, around 10^{-6}, for 19 generations and then gradually fell until only the undiluted cultures in the 88th generation produced a lesion in rabbits.

With the drop in the titer of the virus in the rabbit came a change in the character of the lesions induced by the active agent. The virus in the first 15 generations upon intradermal inoculation produced large edematous lesions with hemorrhagic and necrotic centers. With

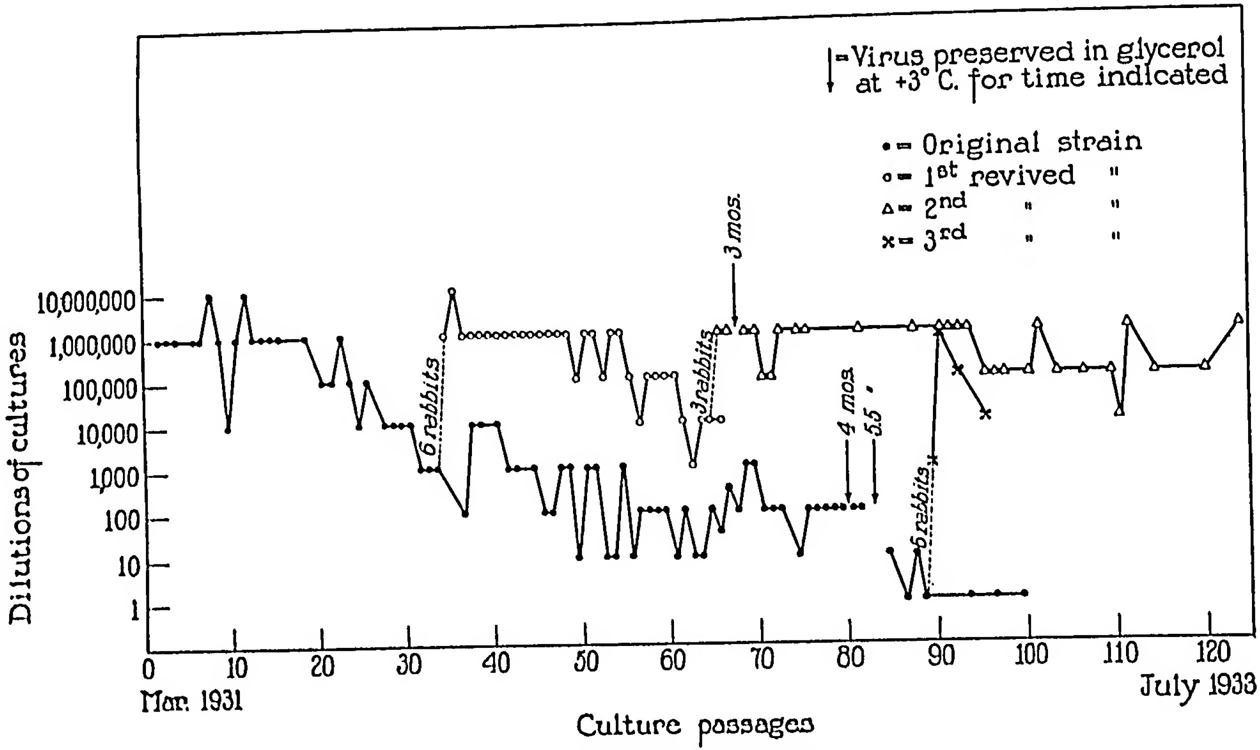

CHART 1. Graphic portrayal of the intradermal titer in rabbits of vaccine virus from different culture passages of the original, 1st revived, 2nd revived, and 3rd revived strains.

material from the 15th to the 70th generations nodular lesions without hemorrhage and necrosis were excited. When the virus was spread on the scarified skin, however, typical vaccinal vesicles appeared. After the 73rd generation, only small flat red areas 1 cm. in diameter were seen at the points of intradermal inoculation of the virus. The erythema faded rapidly and was followed by a superficial scaling of the skin. Upon dermal inoculation of the virus, only a slight amount of redness and scaling was produced which was almost indistinguishable from that caused by scarification alone. In spite of the fact that extremely mild and evanescent lesions were produced in rabbits with material from these cultures, vaccine virus was present in them as will be seen when the reaction induced by them in human beings is discussed.

Revival of the Activity of Culture Virus by Means of Testicular Passages in Rabbits

Inasmuch as vaccine virus for human use is usually tested in rabbits before distribution, we have attempted to obtain a culture strain that retains its pathogenicity both for man and rabbits. Consequently, when the titer of the culture virus for the rabbit began to fall, we passed the virus through several rabbits by means of testicular inoculations. With the passaged virus new cultures were initiated. This procedure has been resorted to three times and the results are detailed below and portrayed in Chart 1.

1 cc. of the pooled 34th generation cultures was injected into each testicle of a rabbit. After 4 days the testicles were removed aseptically and an emulsion was prepared. 1 cc. of this emulsion was then injected into each testicle of another rabbit. This procedure was repeated until the virus had been passed through 6 rabbits. All of the animals had fever. The titer of the virus gradually increased and the intradermal lesions again assumed a hemorrhagic and necrotic character. With testicular virus from the 6th rabbit new cultures were initiated and have been designated as the 1st revived strain. The titer of the first 14 generations of this strain was 10^{-6}, the intradermal lesions were edematous, hemorrhagic, and necrotic. Upon further passage of the virus in cultures the titer gradually fell (Chart 1) and the character of the lesions changed from hemorrhagic and necrotic to nodular.

1 cc. of the pooled cultures of the 31st generation of the 1st revived strain was injected into each testicle of a rabbit. After 4 days the testicles were removed and

an emulsion was made. 1 cc. of this emulsion was injected into each testicle of another rabbit. This procedure was repeated until the virus had been passed through 3 rabbits. All of the animals had fever and typical vaccinal reactions in the testicles. Testicular virus from the 3rd rabbit was used to start a new set of cultures that has been designated as the 2nd revived strain. The titer of this strain has remained around 10^{-6} for 60 culture generations. Although the titer has not fallen to any great extent (Chart 1), the intradermal lesions have become less hemorrhagic and more nodular.

Repeated passages of vaccine virus in the culture medium used in this work appear to decrease the titer of the active agents for rabbits and to mitigate the severity of the lesions produced. We have been able in the manner described above to revive the culture strains by testicular passages in rabbits, and the 2nd revived strain seems to be fairly stable. It may be necessary, however, to repeat the procedure of revival several times more before a completely stable culture virus is obtained.

The original culture strain (Chart 1) was carried for more than 2 years without being revived by passage through rabbits. The 88th culture passage produced little or no reaction in the skin of a rabbit yet produced typical vaccinal reactions in the skin of 2 children. It seemed of interest to find out if this culture could be revived by passages through rabbits.

1 cc. of the pooled cultures of the 88th passage (original strain) was injected into each testicle of a rabbit. The culture virus was also rubbed into the scarified skin and injected intradermally. Only a slight papule appeared at the point of intradermal inoculation and no definite vaccinal lesions were seen in the area of scarification during the 7 day period of observation. The animal had no fever. Nevertheless, the testicles were removed on the 4th day after inoculation and appeared practically normal. An emulsion of the testicles was made and 1 cc. of the emulsion was injected into each testicle of another rabbit. Dermal and intradermal inoculations were also made. The testicles were removed on the 4th day and seemed slightly injected. At the point of intradermal inoculation a lesion, 1 x 1 cm. in diameter, developed and 2 discrete pocks were seen in the area of dermal inoculation. The testicular virus was passed to a 3rd rabbit that developed no fever. The testicles in this animal had the appearance of being affected by a mild vaccinia. At the site of intradermal inoculation a lesion, 6 x 5 cm. in diameter, developed, and 2 discrete pocks were observed in the scarified skin. The 4th rabbit had fever, inflamed testicles, confluent vaccinal eruption at the site of scarification, and an intradermal titer of the virus of 10^{-3}. The 5th rabbit had fever, inflamed testicles, and an intradermal titer of the virus of 10^{-5}. The 6th

rabbit had fever and the testicles were inflamed. From an emulsion of the testicles of the 6th rabbit, cultures were initiated and have been designated as the 3rd revived strain. The titer of the 1st culture was 10^{-3}, of the 2nd 10^{-6}, of the 4th 10^{-5}, and of the 7th 10^{-4} (Chart 1).

From the facts presented above it appears that the 88th culture passage of the original strain of culture virus caused little or no reaction in rabbits. Upon repeated testicular passages in rabbits, however, the virus gradually regained its pathogenicity for that host.

TABLE I

Summary of Results of Vaccinations in Man with the Original Culture Strain of Virus

No. of patients	Culture passage	Preservation of culture		Results	Revaccination with New York City virus
		Time	Manner		
1	5	1 mo.	Glycerol at +3°C.	+	
	10	2 days	" " +3° "	+	
12	11	11 "	" " +3° "	12+	
1	11	2 wks.	" " +3° "	+	–
1	11	3 "	" " +3° "	+	
1	14	6 mos.	Glycerol at –10°C.	–	+
1	14	19 "	" " –10° "	+	–
1	42	1 hr.	Glycerol at +3°C.	+	–
1	42	1 wk.	" " +3° "	+	
1	42	2 wks.	" " +3° "	+	
1	82	1 mo.	" " +3° "	+	–
1	82	39 days	" " +3° "	+	
1	86	1 hr.	Without glycerol at +3°C.	+	–
1	88	1 "	" " " +3° "	+	
1	88	1 "	" " " +3° "	+	–

Jennerian Prophylaxis in Man by Means of Culture Virus

Before discussing the effect that *in vitro* cultivation has on the activity of vaccine virus in man it seems advisable to present in tabular form the results obtained in individuals vaccinated with culture virus. With the original strain, 25 people have been inoculated (Table I). Of these, only 1 failed to develop a typical vaccinal lesion. Six of the individuals who reacted to the culture virus were revaccinated with the New York City Board of Health virus and were found to be refractory. With the 2nd revived strain (Table II), 77 people have

TABLE II

Summary of Results of Vaccinations in Man with the 2nd Revived Culture Strain of Virus

No. of patients	Culture passage	Preservation of culture		Results	Revaccination with New York City virus
		Time	Manner		
1	27_2	1 mo.	Glycerol at +3°C.	+	
1	33_2	2 wks.	Dessiccated	+	
1	36_2	3 "	"	+	
1	42_2	4 days	Glycerol at +3°C.	+	
1	42_2	1 wk.	" " +3° "	+	
1	42_2	11 days	" " +3° "	+	−
1	42_2	1 mo.	" " +3° "	+	−
4	42_2	1 "	" " +3° "	2+ 2−	
5	43_2	2 wks.	" " +3° "	5+	
1	44_2	6 days	Without glycerol at +3°C.	+	
8	44_2	3 wks.	Glycerol at +3°C.	6+ 2−	
11	47_2	2 mos.	" " +3° "	8+ 3−	
1	42_2	1 mo.	" " +3° "	−	
	49_2	4 days	" " +3° "	+	
1	49_2	4 "	" " +3° "	−	
	51_2	1 hr.	Without glycerol at +3°C.	+	
1	49_2	4 days	Glycerol at +3°C.	−	+
	51_2	1 hr.	Without glycerol at +3°C.	−	
2	50_2	1 day	Glycerol at +3°C.	+ 1−	
9	51_2	4 days	" " +3° "	9+	
8	54_2	3 "	" " +3° "	7+ 1−	
19	56_2	3 hrs.	" " +3° "	15+ 4−	

TABLE III

Summary of Results of Vaccinations in Man with the 3rd Revived Culture Strain of Virus

No. of patients	Culture passage	Preservation of culture		Results
		Time	Manner	
16	5_3	4 hrs.	Glycerol at +3°C.	12+ 4−

been vaccinated, 64 of whom developed typical vaccinal lesions. Of those who had reactions, 2 were revaccinated with the New York City Board of Health virus and were found to be refractory. With the 3rd revived strain (Table III), 16 individuals have been inoculated and in 12 of them typical vaccinal reactions occurred. Three children who had been vaccinated with the New York City Board of Health virus were found to be refractory to the culture virus. In summary, 118 individuals have been inoculated with the culture virus and in 100 of them the inoculation was followed by a typical vaccinal pustule. Individuals vaccinated with the culture virus were refractory to a standard dermal strain of calf lymph and *vice versa*. All of the inoculations represent primary vaccinations in infants and children. Approximately one-third of the patients were in the Hospital of The Rockefeller Institute for complete observation during the course of the vaccination. The other two-thirds were vaccinated by us in Dr. Schloss' prophylactic clinic at the Cornell Medical Center and were seen only once after inoculation.

Effect of in Vitro Cultivation on the Activity of Vaccine Virus in Man

With virus from the 5th, 10th, and 11th culture passages of the original strain 15 children were vaccinated. A positive result was obtained in each individual. The reactions were similar to those caused by the New York City Board of Health virus. Consequently, no more children were vaccinated with the virus until it had been passed through 42 successive sets of cultures. Then virus from the 42nd, 82nd, 86th, and 88th sets of cultures were tested in man (Table I). In addition to this, virus from cultures of the 2nd and 3rd revived strains (Tables II and III) were employed for the vaccination of a large number of children. Virus from these cultures did not average as high a percentage of positive reactions as that usually obtained with the New York City Board of Health virus. The reactions, however, were milder than those caused by the Board of Health virus; the children had no fever and were in no way upset. In every respect the results obtained with the culture virus were highly satisfactory.

During the course of the observations it was found that with the later generations of culture virus an area of skin larger than that usually advised had to be scarified in order to insure a positive reaction. Furthermore, it soon became evident that fresh culture virus (Tables I, II, and III) can be used with complete safety. Such is not the case with green calf lymph. Moreover, in view of our experience with the culture virus, we suggest that it be dispensed in cork-stoppered vials containing enough material for 10 vaccinations instead of in capillary tubes containing only sufficient virus for 1 inoculation. This suggestion is made because the culture virus contains a very small amount of particulate matter and it is believed that sooner or later the virus is adsorbed on these particles which in turn tend to adhere to the sides of the capillary tubes. Under these conditions difficulty is encountered in expressing the virus from the tubes and a low percentage of positive reactions is likely to be obtained.

Vaccination by Means of Intradermal Injection of Culture Virus

The results of intradermal vaccinations have been reported by a number of workers. The literature has been fully reviewed in a communication by Roberts (8). In spite of the favorable reports concerning the matter, most physicians have hesitated to use this method because of the fact that very few vaccine virus preparations are entirely free from living bacteria. Inasmuch as we had a bacteria-free virus that caused mild reactions upon dermal inoculation, we decided to see what it would do when injected intradermally into man.

C. B. received intradermally 0.1 cc. of a 1–10 dilution of culture 42_2 that had been preserved for 11 days in 50 per cent neutral glycerol at +3°C. The first signs of a reaction were observed 9 days later and consisted of erythema and a slight amount of induration. The erythema spread and the induration increased for a few days and then gradually disappeared. No pustule formed and no scar was left. The child was not sick or upset and had no fever. Upon revaccination with the New York City Board of Health virus the child was found to be refractory.

R. R. received intradermally 0.1 cc. of a 1–10 dilution of culture 42_2 that had been preserved for 1 month in 50 per cent neutral glycerol at +3°C. The first signs of a reaction were seen on the 7th day after inoculation. The course of events was practically the same as that described for C. B. with the exception that a very small vesicle formed where the needle was inserted in the skin. Upon revaccination the child was found to be refractory.

J. O'B. received intradermally 0.1 cc. of a 1–10 dilution of culture 44_2 that had been preserved without glycerol for 6 days at +3°C. The first signs of a reaction were observed on the 4th day following inoculation. The course of events was the same as that described for R. R. The small vesicle resulted in a minute scar. Upon revaccination the child was found to be refractory.

H. J. received intradermally 0.1 cc. of undiluted fresh culture 88. On the 4th day after inoculation a red papule was seen. The course of events was similar to that of the other children. No pustule formed and no scar was left. Upon revaccination the child proved to be refractory.

From the facts presented above it appears that intradermal vaccination with virus that has been passed through a number of cultures is safe. The virus can be used, undiluted or diluted 1–10, in 0.1 cc. amounts either in the fresh or preserved state.

Effect of Storage on the Titer of Culture Virus

It was essential to determine how well the culture virus withstands storage. Consequently, lots of the same virus were preserved in different ways and later tested in rabbits for potency.

One lot of virus was mixed with an equal amount of 100 per cent neutral glycerol, another lot with an equal amount of 100 per cent neutral glycerol to which heated normal chick embryo tissue had been added, and still another lot with an equal amount of 100 per cent glycerol to which sufficient glucose had been added to make a 2.5 per cent solution. Then these lots of virus were placed in small cork-stoppered vials. Half of each lot of vials was stored at −10°C., the other half at +3°C. From time to time a vial of each lot was removed from storage and the virus was titered intradermally in rabbits.

The results of the work described above are shown in Table IV and indicate that the titer of the culture virus in storage (−10°C. and +3°C.) gradually fell from 10^{-6} but was still 10^{-4} at the end of a year. Furthermore, virus in a vial that had been stored at −10°C. for 19 months produced a typical vaccinal lesion in a child.

Desiccation of the Culture Virus

Vaccine virus does not maintain its activity well in the absence of refrigeration. It has been shown, however, that a number of viruses retain their activity better if they are frozen and then desiccated while in the frozen state. In view of this fact we performed an experiment in which culture virus (36th passage of the 2nd revived strain) was frozen, desiccated, and then stored in sealed tubes at 37°C. Each week for 5 weeks a tube was removed from the incubator, the original volume in the tube was restored with sterile distilled water, and the resulting virus mixture was titered intradermally in a rabbit. The results are brought together in Table V and show that the dried virus maintained some of its activity for 5 weeks even at 37°C. In addition to this fact we have shown (Table II) that desiccated culture virus restored to its original volume with 25 per cent glycerol produces typical vaccinal lesions in human beings.

TABLE IV

Effect of Storage on Titer of Culture Virus (14th Generation of Original Culture Strain)

Time in storage	Stored at −10°C. in cork-stoppered vials			Stored at +3°C. in cork-stoppered vials		
	Equal volume of glycerol added to culture	Equal volume of glycerol added to culture plus extra heated embryo tissue	Equal volume of glycerol added to culture containing 2.5 per cent glucose	Equal volume of glycerol added to culture	Equal volume of glycerol added to culture plus extra heated embryo tissue	Equal volume of glycerol added to culture containing 2.5 per cent glucose
Titer by calculation before storage	500,000		500,000	500,000		500,000
1 day		1,000,000			1,000,000	
1 mo.	100,000	1,000,000	1,000,000	100,000	1,000,000	100,000
3 mos.	1,000,000	1,000,000	1,000,000	100,000	100,000	1,000,000
5 "	100,000	10,000	10,000	10,000	10,000	1,000
1 yr.	10,000	10,000	10,000	10,000	10,000	1,000
19 mos.	E. Ramon +					

TABLE V

Effect of Storage at 37°C. on Desiccated Culture Virus (36th Generation of 2nd Revived Strain)

Time of titration	Intradermal titer in rabbits
Before desiccation	100,000
After desiccation	100,000
After storage for 1 wk. at 37°C	100,000
" " " 2 wks. " 37° "	1,000
" " " 3 " " 37° "	1,000
" " " 4 " " 37° "	1,000
" " " 5 " " 37° "	10

TABLE VI

Initiation of New Cultures from Preserved Cultures

Virus	Time and temperature of preservation	Titer when stored	Amount of dilution in starting cultures	Titer of new series of cultures				
				1st	2nd	3rd	4th	5th
5th generation original strain in glycerol	1 mo. at +3°C.	500,000	1:400	1,100,000	1,000,000	1,000,000		
5th generation original strain in glycerol	3 mos. at +3°C.	500,000	1:400	1,000,000	100,000	100,000		
5th generation original strain in glycerol	10 mos. at +3°C.	500,000	1:400	1,000,000	1,000,000			
8th generation original strain in glycerol	22 mos. at +3°C.	5,000,000	1:400	10,000	10,000		100,000	100,000
43rd generation original strain in glycerol	3 mos. at +3°C.	500	1:400	1	10	1,000	10,000	1,000
6th generation original strain without glycerol	22 mos. at −10°C.	500,000	1:20	100,000	1,000,000		100,000	
33rd generation 2nd revived strain desiccated	5 days at +3°C.	10,000	1:100	100,000	100,000			

Initiation of New Cultures from Preserved Cultures

If the cultivation of vaccine virus is to become a practical procedure, it is essential to know whether new cultures can be initiated with virus from preserved cultures. Therefore, numerous attempts have been made to ascertain the facility with which new cultures can be successfully seeded with virus from cultures that have been frozen and desiccated or from cultures preserved with or without glycerol. We have experienced no difficulty in obtaining fresh cultures in this manner. For convenience a few of the results are shown in Table VI.

DISCUSSION

From the results of the work presented in this paper it is obvious that we have had no difficulty in cultivating a dermal strain of vaccine virus for a period of over 2 years in a medium consisting of bits of viable chick embryo tissue (0.1 gm.) suspended in Tyrode's solution (4–5 cc.) in flasks. This medium was chosen because it is the least likely of all media containing living cells to be contaminated with an unknown or an undesirable virus. It is also evident that culture virus has been successfully employed by us for Jennerian prophylaxis in man. It is hoped that our observations will tempt workers in vaccine virus laboratories to try to adapt this or a similar method of preparation of the active agent for general use. In view of the purity of the virus prepared in this manner and since it causes such mild, yet effective reactions in man, it seems possible that much of the objection to vaccination might be overcome and that the rare but occasional postvaccinal encephalitis might be rendered even more rare, or avoided wholly, by its use instead of that of calf lymph.

Attention should be focussed on the fact that repeated cultivation of vaccine virus in the medium used by us gradually reduced the titer of the active agent for the rabbit and also led to an alteration in the type of lesions produced by the virus in that host. Indeed, the virus that had been cultivated for 2 years in the manner described induced little or no reaction in rabbits. Material from these cultures, however, gave rise to typical vaccinal pustules in man. This observation appears to us to be of importance and among other things seems to indicate that a virus of a desired character for human use can be produced by culture methods. In view of the findings presented at this time, it is believed that the change in the activity of the virus for the rabbit was not due entirely—and perhaps not at all—to a gradual diminution in the amount of virus in successive sets of cultures, but to some alteration in the character of the virus itself.

SUMMARY

A dermal strain of vaccine virus has been passed through 99 successive culture passages. This procedure led to a diminution in the pathogenicity of the active agent for the rabbit. By repeated testicular passages in rabbits, however, the virus regained its pathogenicity for that host. New cultures were initiated with the revived virus. A culture strain of virus that has been twice revived in this manner has remained fairly stable for the rabbit through 60 culture passages and it produces mild, yet effective vaccinal reactions in man.

Virus in early cultures was not attenuated for man, but later cultures of the original strain and cultures of the 2nd and 3rd revived strains produced mild reactions without fever and discomfort to the patients. Intradermal vaccinations with the culture virus are safe and satisfactory.

With the culture virus 118 infants and children have been inoculated and in 100 of them a positive reaction occurred. The culture virus produced a refractory state to a standard dermal strain of calf lymph and *vice versa*.

Culture virus stored in 50 per cent neutral glycerol at −10°C. or at +3°C. maintained a considerable amount of its activity for at least 1 year. Desiccated culture virus sealed in tubes maintained some of its activity when stored at 37°C. for 5 weeks.

Fresh cultures can be initiated without difficulty from desiccated virus or from virus that has been stored with or without glycerol.

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METHODS AND EFFECTS OF INCREASING THE URINARY CONSTITUENTS IN THE BODY

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PLATES 36 TO 39

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The approach to the problem of nephritis, through reducing the amount of functioning renal tissue to the minimum compatible with life, was first made, in 1898, by Bradford (1). The experimental animals died from asthenia and wasting in from 1 to 6 weeks after the excision of three-fourths of both kidneys, but no record was made regarding the gross or histological changes in the remaining bits of tissue. From 1918 to 1926, Allen (2) and his associates studied the phenomena of clinical kidney disease from the standpoint of quantitative deficiency of renal tissue after excising portions of the kidneys and after ligation of the renal vessels. By the removal of three-fourths of the renal tissue these investigators obtained reduction of the concentrating power for urea and other nitrogenous substances; chlorides and ammonia; changes in the acid-base balance; polyuria; heightened blood pressure along with hypertrophy of the remaining kidney tissue and usually of the heart. Apparently no objective information regarding the breakdown of the kidney damaged by operation from functional overstrain was obtained because of the hypertrophy of the remaining renal tissue. Both Bradford's and Allen's experiments fail to throw light on the etiology and progressive nature of chronic nephritis.

In the course of the production of experimental chronic nephritis in dogs with deep x-ray (3), one pole of a kidney occasionally escaped the direct effects of the rays because of screening, and retained enough function to maintain life for from 8 to 12 months. However, the renal insufficiency was invariably progressive and fatal. At autopsy the usual contraction and fibrosis of the renal tissue exposed to x-ray were found, but the screened and protected portions presented a wholly different pathological process (Fig. 1). There was none of the contraction and fibrosis but rather a thick, spongy, yellow cortex in which the glomeruli and tubules were fairly well made out. The blood vessels were of usual thickness. Microscopically the protected portions showed the tubular epithelium swollen and undergoing marked granular, vacuolar, and fatty degeneration. The glomerular tufts were enlarged and congested. In some instances the capillaries were enlarged and hyalinized and the capsular space contained pink staining homogeneous material. In contrast the x-rayed portions showed sclerosis and end-arteritis of the blood vessels, thickened capsules of Bowman, hyalinized glomerular tufts, and extensive replacement of the tubules by fibrous tissue. Obviously the pathological changes in the protected and unprotected portions were of such different character that the etiology was just as different. In other words the changes in the protected portions were not due to x-ray but were progressive, finally resulting in death from renal insufficiency. The progression here was similar to that seen in clinical nephritis and the histological changes seen in the protected pole were not unlike those observed in the better preserved portions of the damaged human kidney in chronic nephritis. The question as to the cause of the granular, vacuolar, and fatty changes seen in the protected poles of the x-rayed kidney arises. Were they the result of functional stress or abuse, associated with the continued abnormally high level of urinary constituents in the blood and tissues?

A more natural and certainly more logical approach to the chronic nephritis problem is to be found not in reducing the amount of renal tissue by operation or x-ray but by producing an excess of normal excretory products in the body and leaving the excretory organs in normal condition. The improvement in animals with chronic nephritis produced by x-ray has been shown, when the meat diet is replaced by bread and milk, by marked reduction of the nitrogen retention, higher carbon dioxide-combining power of the plasma, and reduction of blood pressure. A valuable and practical method of increasing the normal excretory products in the body is the continued use of protein xcesses in the diet. Long and careful investigations have led Newurgh (4), the original and chief exponent of this diet method, to conclude that, "kidney injury is related to those digestion products of protein which vary both quantitatively and qualitatively with the type of protein eaten."

Methods

In seeking a method of confirming and elaborating the etiology and progressiveness of renal insufficiency produced by x-ray nephritis where one pole of the kidney was protected, two procedures have been used. The first of these involved the use of ureteroduodenostomy used by Hinman (5), Hinman and Belt (6), and by Bollman and Mann (7) in combination with deep x-ray applied to the unanastomosed kidney. By the application of deep x-ray to the unanastomosed kidney its functional hypertrophy was prevented and an increasingly high level of urinary constituents was maintained in the body. Ten such experiments were carried out and in all the successful ones marked degeneration was obtained in the anastomosed kidney characterized by granular, vacuolar, and fatty changes in the tubules, especially the convoluted portions, and congestion of the glomerular tufts with hyalinization of the capillaries and homogeneous material in the capsular

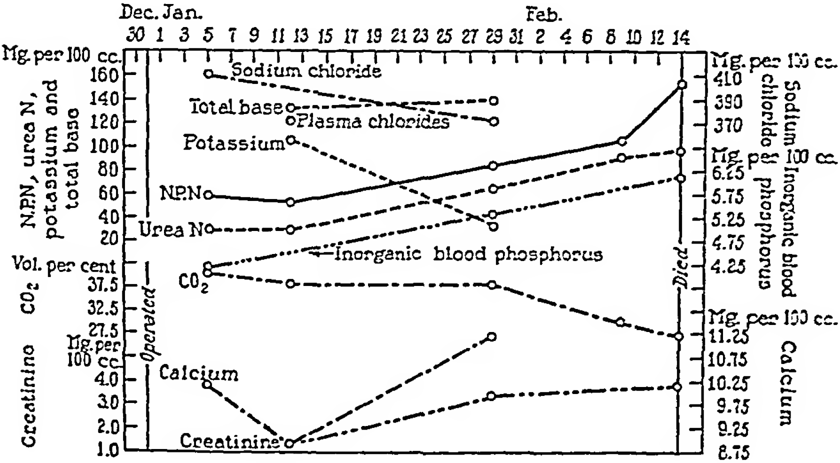

CHART 1. Curves of chlorides, total base, potassium, non-protein nitrogen, urea nitrogen, phosphates, CO_2, calcium, creatinine during 6 weeks from time of cysto-ileostomy to death of animal.

space similar to those found in the protected pole of the x-rayed kidneys. The failures in this group were due to hydronephrosis and infection.

The failures in the first group led to the perfection of a new procedure designed to produce high levels of urinary constituents in the blood without disturbing either the kidneys or ureters. At first this was accomplished by a simple anastomosis between the urinary bladder and the mid-portion of the ileum.

Only young dogs weighing from 15 to 20 kilos, whose urine and blood non-protein nitrogen were normal, were used. All operations were done under ether anesthesia. Later, in order to avoid the gross fecal contamination of the bladder resulting from this procedure, the small bowel was clamped and cut across at the junction of the jejunum and a jejuno-ileostomy was done, leaving a blind length of ileum 18 to 24 inches in length. The fundus of the urinary bladder was then anastomosed to the blind length of ileum near its distal end (Fig. 2). With the latter improvement there was little fecal contamination of the bladder and the urine was suitable for the usual chemical and microscopic analysis.

Results

Both procedures resulted in sustained increase of the level of the urinary constituents in the body and moderate reduction of the carbon dioxide-combining power of the plasma (Charts 1 and 2). After several months the carbon dioxide was more depressed and the urinary constituents higher with eventual death in a uremic phase. The systolic and diastolic blood pressures showed increases of from 10 to 20 mm. of mercury but never reached the high levels obtained in nephritis produced by x-ray. The urine increased in amount as the experiment proceeded and the specific gravity became lower. Albumin was constant in the later stages with as much as 400 mg. per liter in some instances. Examination of the kidneys in various stages showed in the first 6 to 8 weeks a marked enlargement, usually to twice the predicted size. Microscopically the tubular epithelium stained normally but many of the cells were undergoing mitosis, as many as four to six figures often being seen in a single high power field. Beyond the 6 to 8 week period there was no further enlargement of the kidneys but the color usually changed from red or reddish purple to gray or grayish yellow. On section the architecture was less clear-cut. Microscopically, granular degeneration with desquamation of the tubular epithelium and dilatation and congestion of the glomerular tuft capillaries became marked. Still later marked vacuolar and fatty degeneration of the tubular epithelium with actual disintegration and replacement by fibrous tissue became evident. The glomeruli in these later stages showed wide dilatation of the capsular space and filling with homogeneous pink staining material. The tufts were congested or compressed and atrophic (Figs. 3 and 4). The urinary bladder showed the lining intact and the wall of usual thickness. The ureters were of usual size and the walls were of usual thickness. The renal pelves showed no evidence of dilatation or infection so often seen in ureteral anastomosis. Microscopically there was frequently round and wandering cell infiltration of the epithelium and underlying stroma in the bladder. In a few instances similar infiltration was found in the ureter and pelvis of the kidney but there was no extension into the parenchyma. The degenerative changes were similar in the cases showing infiltration and those that did not.

Further Methods and Results

The obvious possibility of ascending infection in both the ureteral and bladder anastomosis procedures led to continued investigation and the development of three additional procedures in which the possibility of contamination and infection from the intestinal tract was reduced. All the operations were done under ether anesthesia.

The first procedure consisted of completely isolating a loop of ileum and at the same time maintaining the continuity of the intestinal tract with a side to side jejuno-ileostomy. The mid-portion of the isolated loop, whose ends were inverted and brought together with a silk suture, was then anastomosed to the fundus of the urinary bladder. The operation was done in one stage under ether anesthesia and the operative mortality was 40 per cent. This procedure was done on five animals, and of the three that survived the operation all showed marked enlargement and degenerative changes in the kidney as described in the cysto-ileostomy group. One animal living 9 months after the operation showed more advanced lesions characterized not only by granular, vacuolar, and fatty changes but in addition by replacement of tubules by fibrous tissue and extensive hyalinization of the glomerular tufts (Fig. 5).

The second procedure consisted of ureteral anastomosis to the gall bladder and subsequent application of deep x-ray to the unanastomosed kidney. The anastomosis to the gall bladder, following the Coffey technic, was done readily because of the thin wall and the tough peritoneal coat. One of the animals showed slight hydronephrosis although there was free drainage into the gall bladder. Neither dilatation nor thickening of the ureter was present. The gall bladder was of usual size and the lining mucosa appeared normal. The anastomosed kidney was larger than usual. The cortex was thicker than normal and grayish yellow in color. Microscopically degeneration of the tubular epithelium was marked and the glomerular tufts showed the characteristic dilatation and engorgement of the glomerular tufts (Fig. 6). The unanastomosed kidneys which had been exposed once directly to deep x-ray and four times through the abdominal wall showed the usual contraction and fibrosis.

The third procedure was devised to allow the administration of urine, urine concentrates, or synthetic salt mixtures into the intestinal tract without disturbing the urinary tract in any way. The simplest and most effective method proved to be to bring a loop of the upper end of the ileum through a short incision in the costovertebral angle under ether anesthesia. A side to side anastomosis was done leaving 8 inches of bowel in the loop and the muscle was closed through the lumen of the loop leaving the intestine immediately beneath the skin. The skin was allowed to heal completely and then a small opening directly through skin and intestinal wall was made for the catheter. The animals were then placed in a sling and the desired solutions administered by the Murphy-drip. For the most part the concentrated human urine prepared and analyzed by Dr. O. H. Gaebler was used. The method of concentration and composition is as follows:

Method of Concentration.—Distillation took place in partial vacuum. The temperature was kept below 50°C. Distillation was discontinued when the urine had been concentrated more than ten times. The residue was made up to one-tenth the original volume of the urine, and filtered from a precipitate of uric acid and earthy phosphates.

TABLE I

	gm.
Ammonia nitrogen	0.29
Urea "	5.95
Creatinine "	0.37
Uric acid "	0.40
Undetermined nitrogen	0.50
Total nitrogen	7.15

Result of analysis, in gm. per 100 cc. of concentrate.

The inorganic salt composition is represented by the following mixture, sodium bicarbonate being used to supply excess of base over chloride, sulfate, and phosphate found.

TABLE II

	gm.
Potassium acid phosphate	0.953
" sulfate	1.498
" chloride	2.288
Magnesium chloride (6 H_2O)	0.264
Calcium chloride (anhydrous)	0.135
Ammonium chloride	1.107
Sodium chloride	6.202
" bicarbonate	1.100
Total inorganic salts	13.545

The results are given in gm. per 100 cc. of concentrate. The solution clouds after a short time with precipitation of earthy phosphate.

From 150 to 250 cc. of concentrate usually produced acute toxic symptoms characterized by drooling of saliva, vomiting, diarrhea, partial suppression of urine, hyperpyrexia, elevation of the erythrocyte count, convulsions, and coma. Seven of the twelve animals in this series succumbed in from 6 to 24 hours during this toxic state. In all the syndrome corresponds closely to that described by Andrews (8). Comparable toxic states were produced by inorganic salt mixtures compounded to resemble the urine concentrates, the same plus urea, and by 50 per cent urea solutions alone. 20 per cent urea solutions have been used by Streicher (9) intravenously for the production of uremia and uremic enteritis. He showed histologically marked parenchymatous degeneration of both liver and kidney.

After the urea solutions the coma was often prolonged for several days and there was more tendency to recovery than with either of the other solutions. The inorganic salt mixture plus urea was more potent in producing the toxic state than either the salt mixture alone or the urea alone. From 275 to 350 cc. of the 50 per cent urea solution were usually necessary to produce convulsions and coma. When a period of from 2 to 3 days was allowed to intervene between administration, the animals seemed to build up a moderate degree of tolerance and survived amounts of the various solutions which were invariably fatal to animals of equal size in the first administration. Of the seven animals which succumbed after the first to third administration the autopsy data were comparable in all.

The viscera were congested and the serous membranes were rather dry. Grayish yellow spots of from 0.5 to 1 mm. in diameter were found over the surface of the liver. However, these were not seen deeper in the parenchyma. The kidneys on section showed the cortex streaked with grayish yellow. The brain was wet and the convolutions were broad and flat. The meninges were for the most part infiltrated with blood. Microscopically the liver showed marked granular and vacuolar changes similar to those described by Andrews and like those seen in our series of cysto-ileostomy experiments. The kidneys showed marked congestion, necrosis, desquamation, and granular degeneration of the tubular epithelium in varying degree. The glomerular tufts were swollen and congested and there was often homogeneous pink staining material and blood in the capsular space (Fig. 7). The meninges showed marked dilatation and engorgement of the blood vessels with extensive blood extravasation throughout. The brain cortex showed engorgement of blood vessels and edema.

The three animals that survived repeated administrations were killed after 4 to 6 months. After the administration of concentrated urine the most marked change in the blood chemistry was the reduction of the carbon dioxide-combining power of the plasma from 35 to 25 volumes per cent and elevation of the chlorides and calcium. The non-protein nitrogen and urea were moderately elevated, averaging 60 and 40 mg. respectively. After the administration of 50 per cent urea solution the non-protein nitrogen and urea rose markedly, the carbon dioxide-combining power of the plasma was sharply depressed as were the calcium and chlorides. The potassium was not markedly depressed as reported by Streicher (9) after intravenous administration.

The gross appearance of the kidney in the animals receiving repeated administration of concentrated urine over a period of months differed from those of animals in the cysto-ileostomy series in that the organs were relatively smaller and dark red in color. Microscopically there was marked granular degeneration and desquamation of the tubular epithelium but also more chronic changes manifest by areas of large dilated tubules lined by flattened epithelium and occasionally containing casts. The glomeruli showed dilatation of the capsular space, dilatation and engorgement of the tuft capillaries (Fig. 8). Prolonged and repeated administration of salt mixtures alone, salt mixtures with urea, and urea alone is now being investigated, but all of these solutions are capable of producing the so called uremic syndrome and acute parenchymatous changes in the kidney.

DISCUSSION

The experimental evidence accumulated from the five different series of procedures designed to increase the normal excretory products in the body indicates that comparable functional and anatomical damage to the kidneys is the usual result. This damage is characterized by granular, vacuolar, and fatty degeneration of the tubules and capillary dilatation, engorgement, and hyalinization in the glomerulus during the earlier stages and in the milder cases. In the later and more severe cases the damage is typified by widespread vacuolar degeneration of the epithelium and dilatation with atrophy or fibrous replacement of the tubules, thickening of Bowman's capsule, dilatation and filling of the capsular space by exudate, and atrophy and hyalinization of the glomerular tuft. No explanation for this damage, which applies equally to all these groups, has been found except the presence of excess of the normal excretory products in the body and their elimination through the kidney. The mechanism of their effect on the renal tissue may also be considered in the light of these experiments. In Group 5 it has been shown that acute parenchymatous destruction of the kidney and often of the liver is produced by the administration of concentrated urine, salt mixtures, or the same plus urea, and urea alone into a loop of small intestine. The necrosis of renal tissue is so extensive in these experiments that it may be readily compared with the effect of mercuric chloride. At lower concentrations, continued over a period of months, an elusive nephrotoxic substance contained in the 0.5 per cent undetermined nitrogen may be considered, but there is no convincing evidence in all the experimental work published thus far. Correlating the suggestion of Andrews (8) and Streicher (9) that the liver is destroyed by the high concentration of salts in the blood and tissues with the finding of similar vacuolar changes seen at times in all of the five groups presented here, it might be concluded that the renal destruction is due to the excretion of this liver protein. This contention may be supported by Newburgh's (10) recent finding that liver is the most potent protein in the dietary production of nephritis. Further, the methods presented here of placing the urinary constituents back into the blood involves direct passage to the liver through the portal circulation. The most striking changes noted in the blood chemistry in the experiments of long duration of all groups is the elevation in non-protein nitrogen and the reduction of the carbon dioxide-combining power of the plasma. However, the acidosis was not severe until late in the process, so that considering MacNider's (11) demonstration of the importance of the acid-base balance in the preservation of the function and anatomical structure of the kidney, it is not felt that acidosis was a large factor for damage. Newburgh and Marsh (12) have shown the importance of amino acids as nephrotoxic substances, especially cystine and tryptophan, but in longer experiments presented here the amino acids of the blood at least were not elevated.

The addition of large amounts of urea by Osborne, Mendel, Park, and Winternitz (13), Addis, MacKay, and MacKay (14), Hinman (15), and Newburgh (10) to an otherwise normal diet planned to increase the excretory work of the kidneys did not result in hypertrophy and caused Newburgh to comment "The increase in size caused by high protein diets is apparently something more than a response to increased activity. It appears to have a more specific cause." Newburgh (16) in a later communication states "It would appear to be true that liver contains forms of non-protein nitrogen other than the purins that are nephropathic." In the various groups of experiments presented here the kidneys responded to the reabsorption of the normal excretory products first with hypertrophy of 100 per cent associated with the presence of many mitoses in the tubular epithelium and then in from 6 to 8 weeks after the maximum hypertrophy had occurred the mitoses disappeared and degenerative changes of progressive nature were noted. Both the amount of hypertrophy and the severity of the renal damage were directly proportional to the degree of increase in the urinary constituents in the body, particularly the non-protein nitrogen. The presence of a nephrotoxic substance in the 0.5 per cent undetermined nitrogen fraction might conceivably account for this, but if such were present the marked hypertrophy found in the early stages would be an unusual physiological response. In summary of all the data it appears that increased excretory work must be considered as a probable major factor in the hypertrophy and subsequent degeneration of the kidneys burdened through reabsorbtion with large excesses of normal urinary constituents.

CONCLUSIONS

1. Five methods for the production of slow continued reabsorbtion of urine in the experimental animal are presented.

2. The effects of this prolonged reabsorption of urinary constituents on the blood chemistry and tissues are shown with special reference to the kidney.

3. Large and rapid increase of urinary constituents in the body is definitely destructive to the kidney.

4. The degenerative changes found in the experimental animal in the protected pole of the kidney showing nephritis produced by x-ray and in man in the unscarred portions of the kidney showing chronic nephritis account for the progressive nature and final renal insufficiency of these conditions and are best ascribed to the high levels of excretory products in the body.

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EXPLANATION OF PLATES

Plate 36

Fig. 1. Photomicrograph, medium power, from protected pole of x-rayed kidney, 13 months after administration of x-ray in animal dying from renal insufficiency, showing marked granular degeneration and desquamation of the epithelium in some tubules with extensive fatty and vacuolar degeneration in others. The nuclei are pyknotic in many instances.

Fig. 2. Urinary bladder, ureters, kidneys, and intestines showing double anastomosis (*a*) between bladder and distal end of ileum and (*b*) between proximal end of ileum and rectum. Duration of anastomosis 2 months with blood non-protein nitrogen of 80 mg. Cortex is thicker than usual with grayish yellow inner zone.

Plate 37

Fig. 3. Photomicrograph, medium power, 4 months after anastomosis. Albumin in capsular space; dilatation and degeneration of capillaries; degeneration and desquamation of tubular epithelium.

Fig. 4. Photomicrograph, medium power, showing degeneration and replacement of tubules 6 months after cysto-ileostomy. The capsular space is greatly dilated and filled with homogeneous pink staining material while the glomerular tuft is small, compressed, and atrophic.

PLATE 38

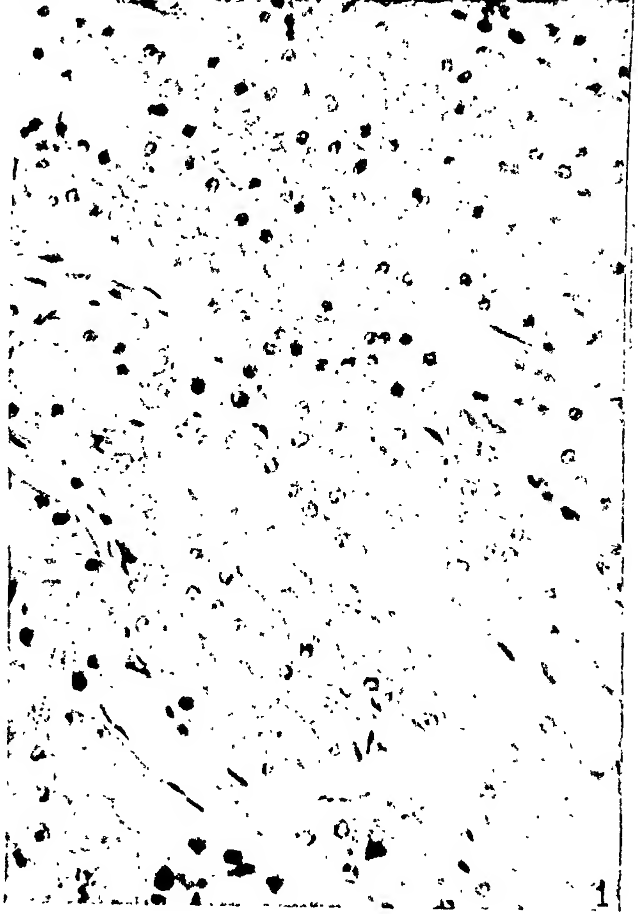
FIG. 5. Photomicrograph, medium power, from kidney where cysto-ileostomy was done. Shows marked degeneration of tubules and also considerable increase in interstitial tissue. The capsular spaces are dilated and the glomerular tufts small and atrophic.

FIG. 6. Photomicrograph, medium power, showing extensive granular and vacuolar degeneration of the tubular epithelium in the kidney after cholecysto-ureterostomy.

PLATE 39

FIG. 7. Photomicrograph, medium power, from kidney of animal succumbing to the administration of 250 cc. of concentrated urine, showing extensive necrosis of the tubular epithelium which resembles the effect of heavy metals.

FIG. 8. Photomicrograph, medium power, from kidney of animal to which concentrated urine had been administered over a 6 month period, showing dilated tubules and granular degeneration of tubular epithelium with desquamation of the same.



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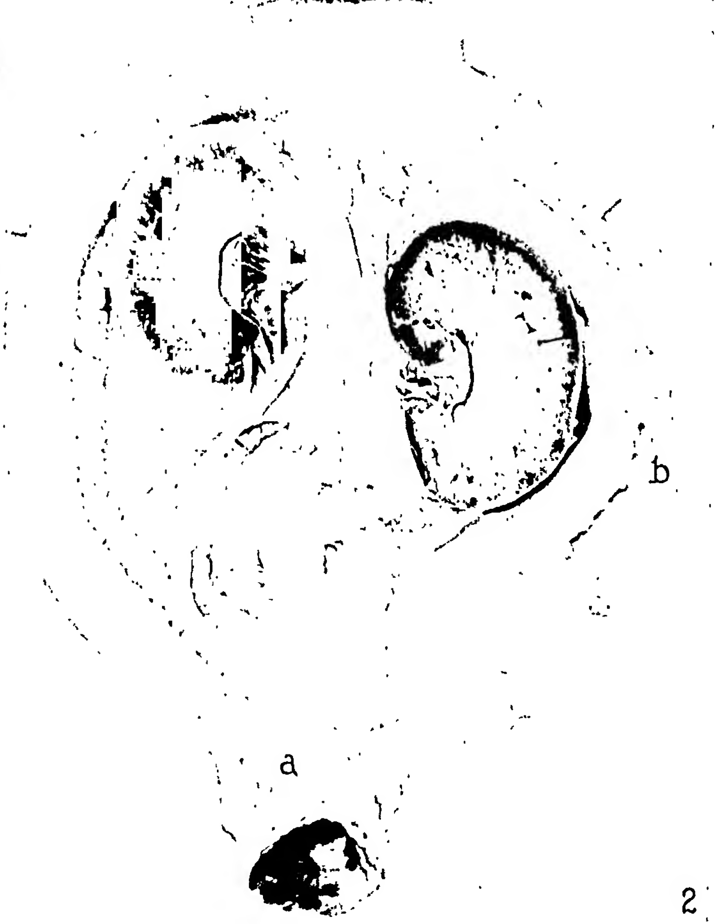

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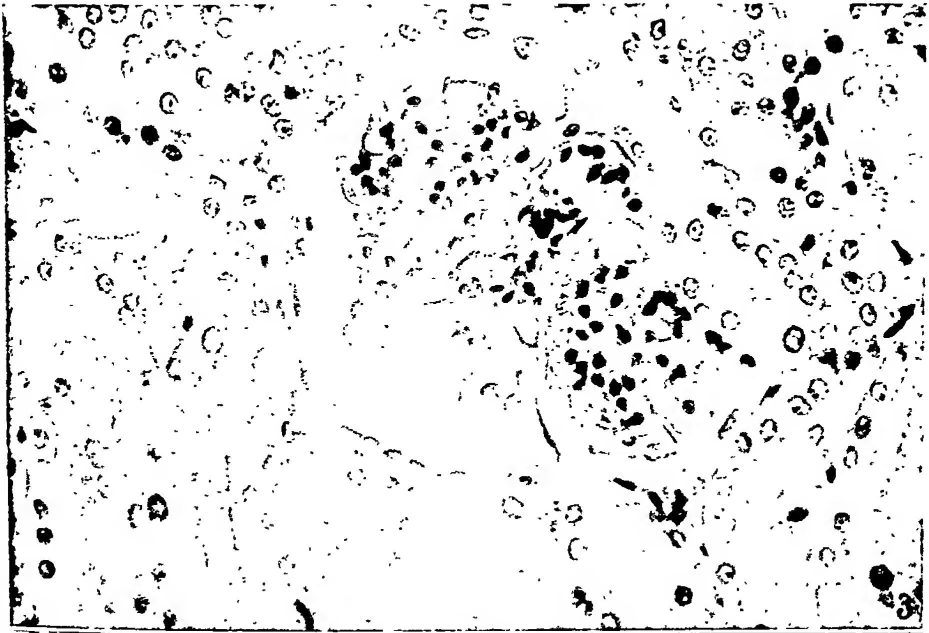

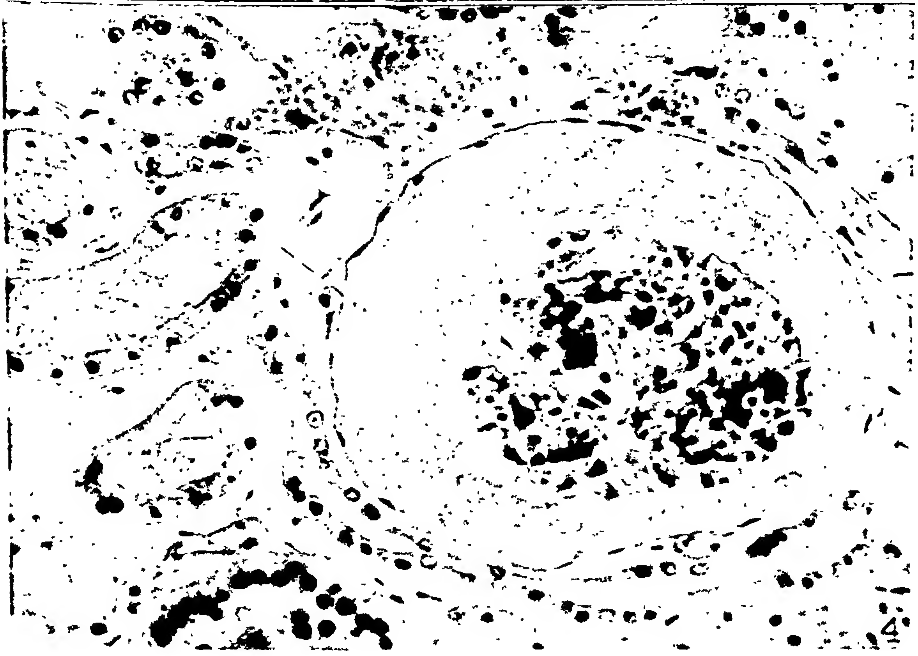

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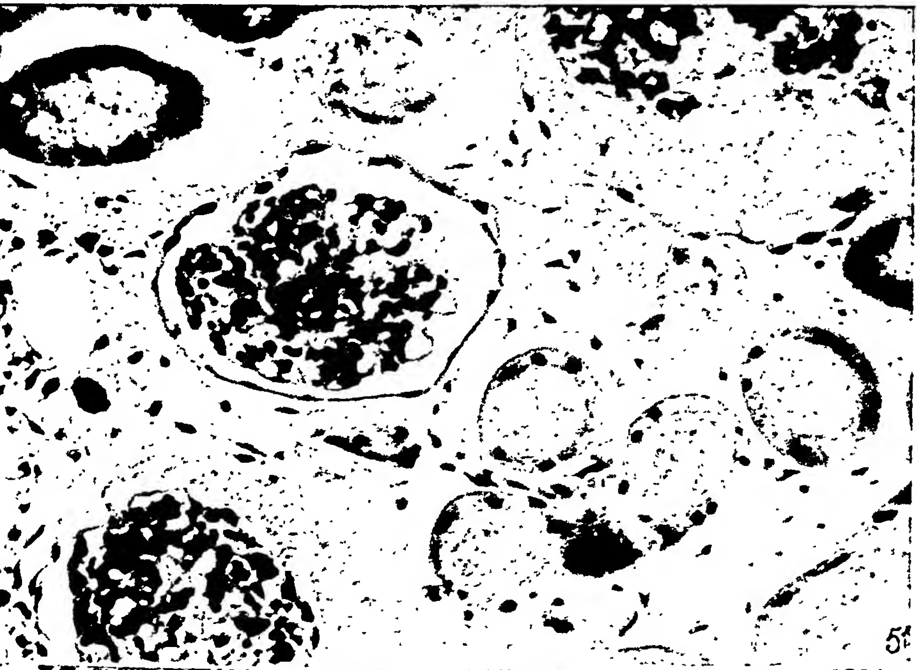

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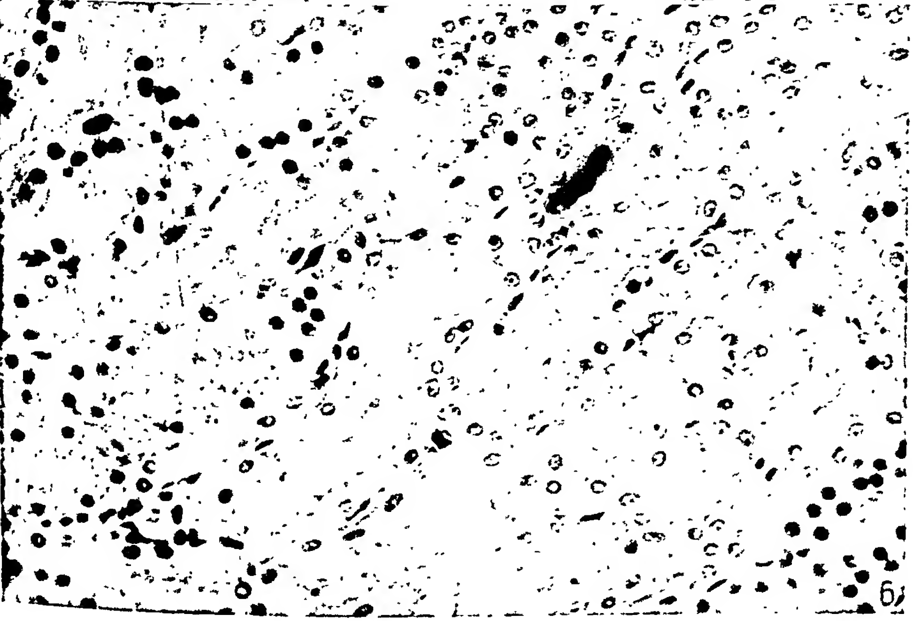

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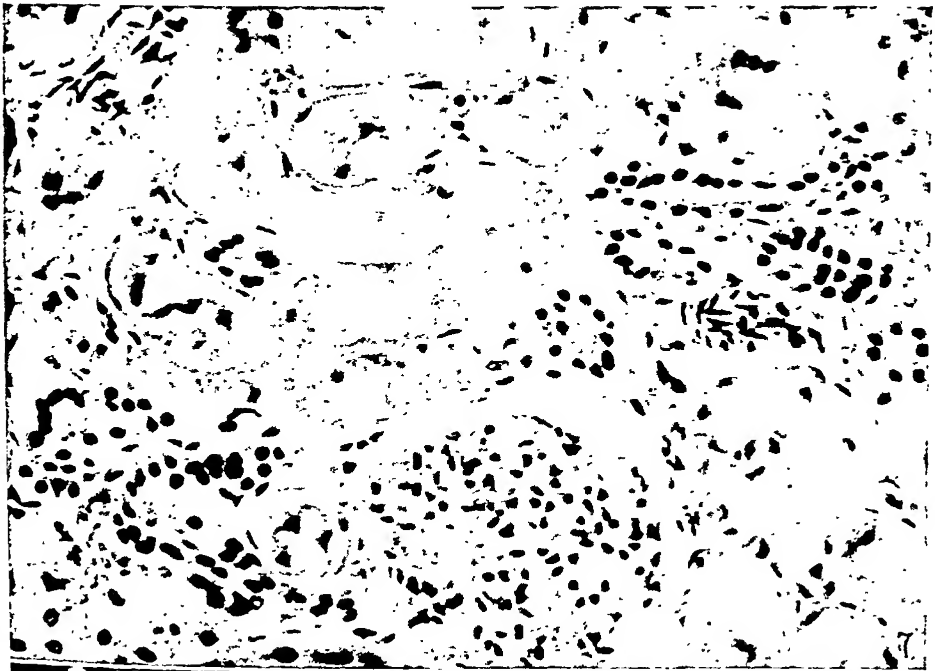

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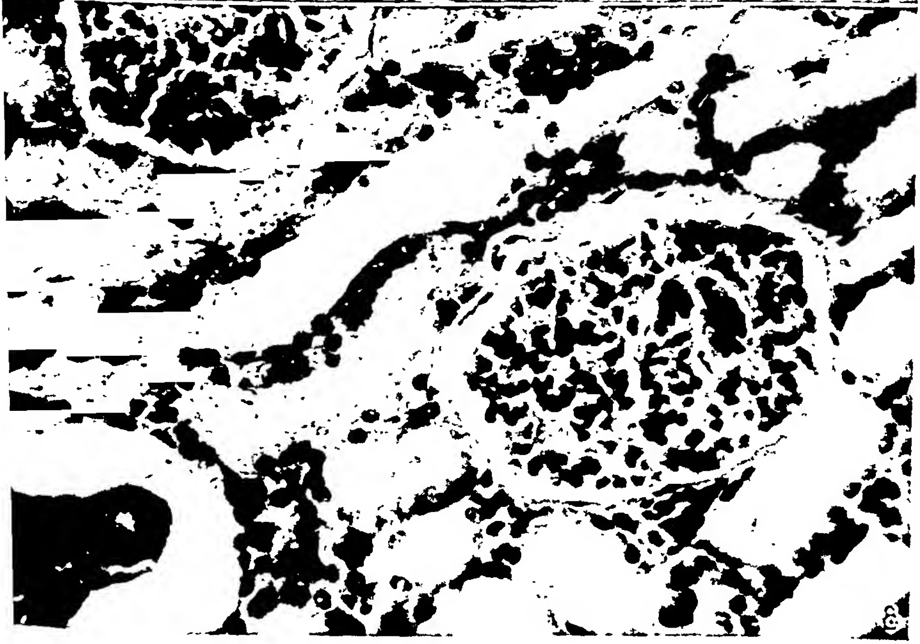

8

(Hartman: Increase in urinary erythrocytes)

CULTIVATION OF PSEUDORABIES VIRUS

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PLATE 40

(Received for publication, July 26, 1933)

All attempts at the cultivation of pseudorabies virus reported in the literature have been unsuccessful. Aujeszky (1) who first described the disease in 1902, Zwick and Zeller (2), Schmiedhoffer (3), Bertarelli and Melli (4), and others were unable to grow the causative agent on lifeless media aerobically or anaerobically. Sangiorgi (5) attempted to cultivate pseudorabies virus by the method devised by Flexner and Noguchi (6) for the cultivation of the globoid bodies in poliomyelitis. The first series of Sangiorgi's cultures was still infectious after 8 days' incubation at 37°C. The first series of subcultures, however, was inactive after 15 days' incubation.

Since pseudorabies virus in all its qualities is a typical representative of the filtrable viruses, some of which have been successfully cultivated in the presence of living cells, an attempt was made to grow it in this manner.

Pathogenic Properties of the Virus Studied

The Hungarian strain of pseudorabies virus was used in our experiments. In 1930 a sample of this strain had been sent to Dr. Shope by Prof. A. Aujeszky of Budapest. Since then the virus has been maintained by rabbit passage and storage in glycerol.

When injected subcutaneously into rabbits and guinea pigs pseudorabies virus causes a regularly fatal disease. Rabbits are considerably more susceptible than guinea pigs. In both species a characteristic symptom constantly present after subcutaneous infection is pruritus, which leads to repeated biting, scratching, and consequent mutilation of the skin at the site of inoculation. The incubation period is from 2 to 3 days and death usually occurs in from 12 to 24 hours after the onset of the first symptoms. More detailed descriptions of the clinical picture in pseudorabies are given by Aujeszky (1), Zwick and Zeller (2), Schmiedhoffer (3), and Shope (7).

While all authors have found both rabbits and guinea pigs susceptible to pseudorabies, their findings in regard to its pathogenicity for mice have been at variance.

Aujeszky (1) found mice to be less susceptible to the virus than rabbits, dogs, and guinea pigs. Schmiedhoffer (3) calls gray mice more susceptible than white ones. Von Rátz (8) could infect white mice by feeding the virus. The incubation period after this mode of infection was very long, 6 to 15 days. In Isobolinski and Patzewitsch's (9) experiments mice were susceptible to pseudorabies. The incubation period in white mice was 3 to 4 days. Zwick and Zeller (2) could not infect white mice by subcutaneous, intramuscular, or intraperitoneal injections of virus. In Shope's (7) experiments with the Iowa ("mad itch") strain of pseudorabies virus white mice developed fatal infections regularly from intracerebral inoculation, irregularly from intraperitoneal infection, and never from subcutaneous injections. Burggraaf and Lourens (10), using pseudorabies virus from an outbreak in Holland in 1932, could not infect mice by intraperitoneal or subcutaneous injection. Unless there are variations in the susceptibility of white mice used by different laboratories to pseudorabies virus the different results obtained by the authors mentioned above, who worked largely with the Hungarian strain, cannot be explained.

Since it was intended to use white mice for the titration of the virus, it was necessary to know whether our mice were regularly susceptible to the Hungarian strain of pseudorabies virus or not.

In a preliminary experiment it was found that white mice die regularly in 3 to 4 days after a sufficiently large dose of virus administered intraperitoneally. The intraperitoneal route of infection was preferred to the intracerebral one, since a greater amount of fluid could be injected in this way and therefore the dosage could be regulated more accurately.

The disease in mice was found to be as constantly fatal as in guinea pigs and rabbits. When approximately 9/10 of the inoculum was injected intraperitoneally and 1/10 subcutaneously at the same site, 70 to 80 per cent of the mice showed the symptoms characteristic in rabbits and guinea pigs after subcutaneous injection: active biting and scratching, leading to self-mutilation at the site of inoculation or some other place on the body surface. The mice which failed to develop pruritus showed a greatly accelerated respiratory rate, dyspnea, and salivation. After some experience with the disease in mice it is not difficult to establish the clinical diagnosis in every case.

In Table I the results of a titration of rabbit brain pseudorabies

subcutaneously at the same site. When, for comparison, the titer of the fluid and tissue portions of cultures was determined, they were centrifuged, following which the supernatant fluid was pipetted off and divided into two equal parts. The tissue was ground with sand and one-half of the supernatant fluid was added to obtain a suspension which could be injected. Decimal dilutions of this suspension and of the other half of the supernatant fluid were tested in mice. As a rule, several cultures of a serial passage were used for titration to lessen the effect of variations in virulence of single cultures. The results of the cultivations are given in Table II.

The virus was cultivated uninterruptedly for 12 serial passages. The cultures of Series XIII were inactive when tested in mice. In this series media were used which had been stored in the refrigerator for 6 days prior to inoculation. It is likely that the cells in the medium had died during this time and were no longer suitable for the multiplication of virus.

To determine whether the virus had actually disappeared from the cultures or whether its pathogenic properties for mice had been altered, subcultures were made and tested in guinea pigs and rabbits by subcutaneous and intracerebral inoculation. These animals developed no illness. The guinea pigs, when tested for immunity 3 weeks later, succumbed to the disease.

A new set of cultures (Xa) was inoculated from the cultures of Series IX which had been stored in the refrigerator for 21 days after incubation and proven to be still virulent for mice. The virus was then carried through 20 further serial culture passages. The cultures of Series XXXI were contaminated by bacteria and avirulent for mice. Therefore a new series of cultures (XXIXa) was started from Series XXVIII, the cultures of which had been kept in the refrigerator for 5 days. After the virus had been cultivated in 4 more serial passages the experiment was discontinued.

The titer of the serial cultures fluctuated considerably, as Table I shows. The reason for this fluctuation is not known. It is noteworthy that, while 1 cc. of the cultures of Series XXIX failed to infect a guinea pig by subcutaneous inoculation, 0.4 cc. of the same cultures contained enough virus to infect subcultures. The tissue portion of the cultures of Series XXV reached the titer of at least 1:1 million, which is almost incredibly high for pseudorabies. In all cultures fractionally titrated the tissue portion contained consid-

(Andrewes (12, 13)), and herpes virus (Andrewes (14)) can be cultivated *in vitro* in series in minced rabbit testicle suspended in rabbit serum and Tyrode solution.

Preparation of Media Used

The testes of a healthy adult rabbit were removed aseptically, washed twice in physiological salt solution, and finely minced with long scissors on a watch-glass contained in a Petri dish. The tissue pulp in amounts of 100 to 150 mg. was distributed with a large loop or by wide mouth pipettes to 50 cc. Florence flasks. The flasks were closed with cotton plugs covered with a layer of tin-foil. As a rule, 2 cc. of rabbit serum (mixtures of sera from several normal rabbits were used) and 2 cc. of Tyrode solution prepared according to the formula given by Fischer (15) were added at once to the tissue pulp. The flasks were then slightly shaken to distribute the tissue fragments equally in the fluid. Sterilization of the serum and Tyrode solution had been effected by passage through Berkefeld N or W filters. The cultures of each serial passage were made up in duplicate or triplicate. From the testes of every rabbit killed for tissue, enough media for two or three culture series was usually made up. Uninoculated culture flasks were stored in the refrigerator, at approximately +4°C., until needed.

Course of the Serial Cultivation

Each flask of Culture Series I was inoculated with 0.3 cc. of a 10 per cent brain emulsion from a rabbit that had died of pseudorabies following intracerebral inoculation. Subcultures were inoculated with 0.4 cc. of the cultures of the preceding series. Thus the dilution factor in the serial culture passages was about 11. The inoculum usually contained a number of small tissue fragments which were drawn into the pipette with the fluid. After inoculation the flasks were either put into the refrigerator for 1 hour according to the method of Carrel (16), or directly into the incubator after it had been found that storage in the refrigerator prior to incubation was not necessary. This observation agrees with Andrewes' (13) findings concerning Virus III. Incubation was carried out at 37°C. for 2 days unless otherwise stated. After incubation the cultures were tested for sterility and contaminated cultures were discarded. The presence of the virus in cultures was determined by subcutaneous injection of 1 cc. of cell-containing culture fluid into a guinea pig or a rabbit.

The titer of the cultures was determined by injecting decimal dilutions of whole ground cultures or culture fractions into white mice. Before the titration of whole cultures the fluid and tissue were carefully separated. The tissue fragments were ground with a small amount of sand in a mortar and then the fluid portion slowly added. From the suspension thus obtained decimal dilutions were made with physiological salt solution. A fresh pipette was used for every dilution, 1 cc. of which was injected into one or two mice, 9/10 of the dose intraperitoneally, 1/10

subcutaneously at the same site. When, for comparison, the titer of the fluid and tissue portions of cultures was determined, they were centrifuged, following which the supernatant fluid was pipetted off and divided into two equal parts. The tissue was ground with sand and one-half of the supernatant fluid was added to obtain a suspension which could be injected. Decimal dilutions of this suspension and of the other half of the supernatant fluid were tested in mice. As a rule, several cultures of a serial passage were used for titration to lessen the effect of variations in virulence of single cultures. The results of the cultivations are given in Table II.

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subcutaneously at the same site. When, for comparison, the titer of the fluid and tissue portions of cultures was determined, they were centrifuged, following which the supernatant fluid was pipetted off and divided into two equal parts. The tissue was ground with sand and one-half of the supernatant fluid was added to obtain a suspension which could be injected. Decimal dilutions of this suspension and of the other half of the supernatant fluid were tested in mice. As a rule, several cultures of a serial passage were used for titration to lessen the effect of variations in virulence of single cultures. The results of the cultivations are given in Table II.

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TABLE II

Cultivation of Pseudorabies Virus in Rabbit Testicle Medium

No. of serial culture passage	Date	Test for virulence by subcutaneous inoculation				Titer* of culture virus for mice			Length of time media was stored in refrigerator before inoculation
		Rabbits		Guinea pigs					
		No.	Result	No.	Result	Fluid portion of cultures	Tissue portion of cultures	Whole ground cultures	
	1933								*days*
I	Feb. 15	•†	•	•	•	1:10	•	•	0
III	" 19	574	+ 56 hrs.	•	•	•	•	•	0
V	" 28	652	+ 71 "	769	+ 55 hrs.	•	•	•	0
VIII	Mar. 6	•	•	768	+ 70 "	•	•	1:100	2
IX	" 8	•	•	789	+ 62 "	•	•	•	4
XII	" 14	•	•	805	+ 95 "	•	•	•	4
XIII	" 16	•	•	•	•	•	•	Avirulent	6
Xa (inoculated with 0.4 cc. of IX)	Apr. 1	•	•	821	+ 61 hrs.	•	•	•	0
XIIa	" 5	•	•	820	+ 64 "	•	•	1:1,000	4
XIIIa	" 7	•	•	828	+ 64 "	•	•	1:1,000	0
XIIIa	" 7	•	•	834	+ 67 "	•	•	1:1,000	0
XIV	" 9	•	•	822	+ 64 "	•	•	1:1,000	2
XIV	" 9	•	•	826	+ 67 "	•	•	1:1,000	2
XVI	" 13	•	•	855	+ 72 "	•	•	1:1,000	0
XX	" 21	•	•	841	+ 60 "	1:100	1:10,000	•	2
XXI	" 23	•	•	860	+ 92 "	•	•	Avirulent	4
XXIV	" 29	•	•	876	+ 46 "			1:10,000	0
XXV	May 1	•	•	839	+ 67 "	1:100	1:1 million	•	2
XXVII	" 5	•	•	881	+ 57 "	Avirulent	1:100	•	0
XXVIII	" 7	•	•	883	+ 66 "	•	•	•	2

XXIX	" 10	•	•	884	—	•	•	Avirulent	5
XXX	" 12	•	•	891	+ 99 hrs.	•	•	•	0
XXXI (contaminated)	" 14	•	•	•	•	•	•	Avirulent	2
XXIXa (inoculated with 0.4 cc. of XXVIII)	" 14	•	•	895	+ 70 hrs.	•	•	•	2
XXXa	" 16	•	•	889	+ 57 "	•	•	•	0
XXXIa	" 18	•	•	•	•	Avirulent	•	•	0
XXXII	" 20	•	•	•	•	•	•	1:10	4

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* By titer in this table and the following ones is meant the highest decimal dilution, 1 cc. of which killed mice when inoculated 9/10 intraperitoneally and 1/10 subcutaneously.

† • = not tested; + = died after; — = no illness.

erably more virus than the fluid portion (see Series XX, XXV, XXVII, and XXXIa in Table II). The tissue could not be freed from virus by repeated washing.

In Table III the results of the titration of the cultures of the last series with rabbit testicle tissue are given as an example of the titration of cultivated virus.

Various factors governing the growth of the pseudorabies virus in cultures were studied in the following experiments.

TABLE III

Titration of Cultures of Series XXXII in Mice

Mouse No. (Series 33)	Dose (ground culture suspension)	Result
	cc.	
1	1.0	Died in 79 hrs.
2	0.1	" " 90 "
3	0.01	" " 104 "
4	0.01	No illness
5	0.001	" "
6	0.001	" "
7	0.0001	" "
8	0.0001	" "

Amount of Tissue Required

Rivers (17)[1] and Rivers and Ward (18) observed that too large amounts of tissue in cultures of vaccinia virus inhibited or prevented the multiplication of the virus. For pseudorabies virus the same seems to be true, although this virus requires a somewhat greater amount of tissue than does vaccinia virus. In Experiment 4 (Table IV) the virus did not multiply in Culture 1 containing 805 mg. tissue, whereas the titer of the control culture (135 mg. tissue) inoculated from the same source was at least 1:1,000. In cultures which contained from 12 to 270 mg. tissue, and which were inoculated with the usual amount of culture material, the virus readily multiplied. A

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relationship seems to exist between the amount of tissue in a culture and the amount of inoculum used in infecting it: the smaller the amount of inoculum, the greater the amount of tissue required in the culture to insure infection (Experiments 1 and 2, Table IV).

Amount of Serum Required

That rabbit testicle culture medium must contain serum in order to support growth of pseudorabies virus was shown by three unsuccessful attempts to grow the virus in rabbit testicle tissue suspended in Tyrode solution without rabbit serum, according to the method of Li and Rivers (19).

TABLE IV

Influence of the Amount of Tissue in Cultures on the Multiplication of Pseudorabies Virus

Experiment No.	Inoculum cc.	Inoculum Culture passage	Culture No.	Amount of tissue	Titer for mice
				mg.	
1	0.1	IX	1	130	1:100
			2	50	Avirulent
			3	10	"
2	0.1	XIIIa	1	270	<1:1,000
			2	140	1:100
			3	10	1:10
	0.4	XIIIa	4	210	1:1,000
			5	82	1:1,000
			6	12	1:1,000
3	0.4	XIa	1	180	1:1,000
			2	90	1:100
			3	10	1:100
4	0.4	XV	1	805	Avirulent
			2	135	<1:1,000

In the first experiment a medium consisting of 100–150 mg. minced rabbit testicle suspended in 4 cc. Tyrode solution was inoculated with 0.5 cc. of a 10 per cent suspension of virulent rabbit brain. After the cultures had been incubated for 3 days at 37°C. they were avirulent for guinea pigs.

erably more virus than the fluid portion (see Series XX, XXV, XXVII, and XXXIa in Table II). The tissue could not be freed from virus by repeated washing.

In Table III the results of the titration of the cultures of the last series with rabbit testicle tissue are given as an example of the titration of cultivated virus.

Various factors governing the growth of the pseudorabies virus in cultures were studied in the following experiments.

TABLE III

Titration of Cultures of Series XXXII in Mice

Mouse No. (Series 33)	Dose (ground culture suspension)	Result
	cc.	
1	1.0	Died in 79 hrs.
2	0.1	" " 90 "
3	0.01	" " 104 "
4	0.01	No illness
5	0.001	" "
6	0.001	" "
7	0.0001	" "
8	0.0001	" "

Amount of Tissue Required

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			6	12	1:1,000
3	0.4	XIa	1	180	1:1,000
			2	90	1:100
			3	10	1:100
4	0.4	XV	1	805	Avirulent
			2	135	<1:1,000

In the first experiment a medium consisting of 100–150 mg. minced rabbit ticle suspended in 4 cc. Tyrode solution was inoculated with 0.5 cc. of a 10 per t suspension of virulent rabbit brain. After the cultures had been incubat 6 days at 37°C. they were avirulent for guinea pigs.

In the second experiment culture virus[2] was used as inoculum for the 1st series of cultures. The virus was still demonstrable in the 2nd series of subcultures. The 4th, 5th, and 6th series of subcultures were avirulent. The virus could apparently multiply in the cultures of the first 2 series because they contained traces of serum from the inoculum. When the amount of serum introduced in this way had reached a certain dilution, multiplication of the virus was no longer possible. In control cultures containing 2 cc. rabbit serum and 2 cc. Tyrode solution the virus readily multiplied.

TABLE V

Influence of the Ratio of Rabbit Serum to Tyrode Solution in Cultures on the Multiplication of Pseudorabies Virus

Culture series	Ratio of serum to Tyrode solution	Inoculum		Test for virulence and titer of serial passages			
		cc.	Culture passage	1st passage	2nd passage	3rd passage	4th passage
a	1:1	0.4	XX	•*	•	•	<1:1,000
b	1:3	0.4	XX	1 cc. s.c. Guinea Pig 870. Died in 58 hrs.	1 cc. i.p. in mouse. Died in 51 hrs.	•	<1:1,000
c	1:9	0.4	XX	•	•	•	1:100
d	1:49	0.4	XX	•	•	•	1:100
e	1:99	0.4	XXV	1 cc. i.p. in 2 mice. Died in 92 and 112 hrs.	•	1 cc. i.p. in 2 mice. No illness	
f	1:999	0.4	XXV	1 cc. i.p. in mouse. Died in 68 hrs.	•	1 cc. i.p. in 2 mice. No illness	
g (control for e and f)	1:3	0.4	XXV	•	•	1 cc. i.p. in mouse. Died in 93 hrs.	

* • = not tested; s.c. = subcutaneously; i.p. = intraperitoneally.

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[2] By "culture virus" is meant pseudorabies virus that has been grown *in vitro* by the methods of cultivation described. The term "culture virus" is used in distinction to the term "brain virus" by which is meant pseudorabies virus from the brain of an animal dead following cerebral infection.

The third experiment was carried out similarly. The 1st series of subcultures was still virulent; the 2nd series, however, was avirulent.

An attempt was then made to determine the ratio of serum to Tyrode solution most favorable to the multiplication of the virus. The results of these experiments are recorded in Table V.

The virus multiplied best in cultures in which the ratio of serum to Tyrode solution was 1:1 and 1:3. In cultures in which this ratio was 1:99 and 1:999, the virus could not be cultivated in series, although it multiplied in serial control cultures containing 1 part serum to 3 parts Tyrode solution inoculated from the same source. During the serial cultivation of pseudorabies virus the impression was gained that 1:1 represented a more favorable proportion of serum to Tyrode solution than 1:3.

TABLE VI

Influence of Time of Incubation of Cultures on Multiplication of Pseudorabies Virus

Experiment No.	Inoculum		Length of time incubated	Titer for mice
	cc.	Culture passage		
			days	
1	0.4	XIIa	2	1:1,000
			3	1:100
			4	1:100
2	0.4	XV	2	1:1,000
			3	1:1,000
			4	Avirulent
			5	"
			6	"
3	0.4	XXIII	1	1:10
			2	1:100
			3	1:1,000
			4	Avirulent
			5	"
			6	"

Time of Incubation

A number of cultures containing equal amounts of fresh tissue, 2 cc. rabbit serum, and 2 cc. Tyrode solution were inoculated from the same source and titrated in mice after various periods of incubation at 37°C. The results are shown in Table VI.

In Experiments 1 and 2 (Table VI) the cultures had reached their maximum virus content after 2 days' incubation and in Experiment 3 after 3 days' incubation. Incubation of from 4 to 6 days inactivated the cultures in Experiments 2 and 3; this was probably due, as is shown later, to the necrosis of the tissue. For the cultivation of the virus in series 2 days' incubation proved to be sufficient.

Cultivation in Long Test-Tubes

Maitland, Laing, and Lyth (20) found that while vaccinia virus could be grown in Carrel flasks in which the depth of the medium was from 1 to 2 mm., it did not multiply in test-tubes when the height of the column of the fluid covering the tissue was 15 mm. Under such conditions the respiration of the tissue, as measured by the method of Warburg, ceased after 24 hours' incubation at 37°C. The authors believed that there was a correlation between the multiplication of the virus and the respiratory activity of the cells contained in the medium.

The growth requirements of pseudorabies virus differ from vaccinia virus since multiplication was demonstrated in 100–150 mg. minced rabbit testicle tissue suspended in 3 cc. Tyrode solution and 1 cc. rabbit serum in long test (Noguchi) tubes, in which the column of fluid overlying the tissue was from 5–6 cm. high.

Length of Time Media May Be Stored in Refrigerator before Inoculatio

The question of how long minced rabbit testicle tissue suspende in 2 cc. rabbit serum and 2 cc. Tyrode solution could be stored in the refrigerator before inoculation was of importance in these experiments. In almost every case the tissue was still suitable for the multiplication of virus after it had been stored in the refrigerator for 4 days prior to inoculation. In one case (see Culture Series XXIX, Table I) the virus did not multiply in tissue which had been kept in the refrigerator for 5 days. In Culture Passage XIII a medium was used which had been stored in the refrigerator for 6 days. The virus became inactive in this medium after 2 days' incubation. In four other experiments media stored in the refrigerator for 6 days were tested. In two of these cases the virus was no longer demonstrable after 2 days' incubation, in the other two cases there was slight multiplication of the virus.

In culture media which had been in the refrigerator for 7, 8, 9, and 10 days prior to inoculation with virus, the virus did not multiply. Such cultures were inactive after 2 days' incubation. In all these experiments the virus multiplied in control media which were fresh or had been kept in the refrigerator for from 1 to 4 days. The failure to grow in stored media is probably due to death of the tissue.

Time of Survival of Culture Virus in the Refrigerator

Cultures of known virulence were stored in the refrigerator for various periods of time and then again tested for their virulence for mice. All cultures were still active after they had been kept in the refrigerator for from 1 to 15 days; after 19 days one culture out of two was avirulent; after 21 days three out of three cultures were still virulent. All cultures which had been in the refrigerator longer than 21 days were inactive. Subcultures could be made from cultures which had been stored in the refrigerator for 1, 2, 4, 5, 16, and 21 days.

Histological Examination of Culture Tissue

Freshly prepared culture media (100–150 mg. minced rabbit testicle suspended in 2 cc. rabbit serum and 2 cc. Tyrode solution) were inoculated with culture virus as usual and incubated for 1, 2, 3, or 4 days. As controls, uninoculated media from the same source were incubated simultaneously for the same period of time. After incubation, pieces of tissue from the culture and control flasks were fixed in Zenker's or Allen's fluid. Paraffin sections were made and stained with phloxin-methylene blue or hematoxylin-eosin. Mouse inoculations in every case showed that virus was present in those cultures from which pieces of tissue had been removed.

Sections from cultures incubated for 1 day showed slight necrosis of the epithelium and very little necrosis of the interstitium. In some interstitial cells the nuclear membranes were slightly hyperchromatic. No inclusions were seen.

In sections from cultures incubated for 2 days necrosis was more pronounced. The interstitium still looked fairly healthy, especially at the edge of the sections. Acidophilic intranuclear inclusions appeared in the interstitial cells of Leydig, in endothelial cells of capillaries, and in connective tissue cells of the lamellar membranes of the tubules (Fig. 1). These inclusions resembled those described by Hurst (21) in many different kinds of cells of rabbits infected with pseudorabies. They were irregular in size, and, sometimes, in shape. Some-

serum and Tyrode solution, four attempts were made to grow pseudorabies virus under similar conditions. In two of these experiments, cell-free extracts of rabbit brain and rabbit testicle were added to the medium containing approximately 100 mg. minced rabbit kidney in 2 cc. rabbit serum and 2 cc. Tyrode solution. The same technique was followed as for the cultivation of the virus in testicular tissue. In none of the four experiments did pseudorabies virus multiply.

Two experiments with minced rabbit liver and one experiment with rabbit blood were equally unsuccessful.

It may be recalled at this time that Andrewes (13) was unable to grow Virus III in media with minced rabbit kidney and liver as tissue constituents.

Cultivation of Pseudorabies Virus in Guinea Pig Testicle Media

After several unsuccessful attempts pseudorabies virus was cultivated in series in minced testicle tissue taken from guinea pigs of about 500 gm. in weight and suspended in 1 cc. guinea pig serum + 3 cc. Tyrode solution. This ratio of serum to Tyrode solution appeared to be favorable to the multiplication of the virus. Virus cultivated in chick embryo media (see below) was used to infect the cultures of the first series. After the 6th serial passage of the virus through the guinea pig medium the experiment was discontinued. When rabbit serum was substituted for guinea pig serum in this medium, the virus failed to multiply. The pathogenic properties of pseudorabies virus cultivated in guinea pig testicle medium did not differ from that in rabbit testicle medium. The possibility of cultivating pseudorabies virus in guinea pig testicle tissue will be made use of later, when an attempt will be made to grow the virus in tissue from guinea pigs immune to the disease.

Cultivation of Pseudorabies Virus in Chick Embryo Media

Ten day chick embryos were finely minced with scissors, after their eyes had been removed, and 100–150 mg. of the tissue pulp was suspended in 3 cc. Tyrode solution and 1 cc. sheep serum. The same technique was followed as for the cultivation of the virus in rabbit testicle tissue. Virus from the 30th culture series in rabbit testicle medium was used to start the cultures, and the results of the experitimes they had well defined margins. Some nuclei contained several small inclusions, others a single large one. In inclusion-bearing cells and many others hyperchromatosis of the nuclear walls was constantly present; such hyperchromatosis appeared also in sections from uninoculated controls, but in them inclusions were never seen. When the inclusions were completely formed, there was a narrow free space between them and the nuclear membrane. In cells of the seminiferous epithelium inclusions were never found.

In cultures incubated for 3 days there was considerably more necrosis of the tissue. Inclusions were still present.

The tissue of cultures incubated for 4 days was generally necrotic. The nuclei of the cells in which inclusions usually appeared were karyorrhectic. Inclusions were no longer distinct (Fig. 2). In sections from uninoculated control media the interstitium, after 4 days' incubation, still looked surprisingly healthy (Fig. 3). The fact that tissues from cultures were always much more necrotic than those from uninoculated controls is attributed to the action of the virus.

Intranuclear inclusions appeared in only about 60 per cent of the pseudorabies cultures examined histologically. The reason for this irregularity may be that, in some cultures, not all of the many small pieces of tissue become infected by the virus, and that uninfected tissue particles were picked out in those cases in which inclusions were not found.

Inclusions of the same type and in the same kind of cells were also found *in vivo* in the testis of a rabbit that had been infected intratesticularly with virus from rabbit brain. The inclusions were completely formed 24 hours after inoculation, that is, during the incubation period.

Many of the inclusion bodies in pseudorabies cultures are somewhat similar to those found by Andrewes (12) in rabbit testicle cultures of Virus III. The Virus III inclusions, however, appeared in interstitial cells exclusively.

Attempts to Cultivate Pseudorabies Virus in Rabbit Kidney, Liver, and Blood

Since vaccinia virus was found by Maitland and Laing (11) and others to multiply in minced rabbit kidney tissue suspended in rabbit serum and Tyrode solution, four attempts were made to grow pseudorabies virus under similar conditions. In two of these experiments, cell-free extracts of rabbit brain and rabbit testicle were added to the medium containing approximately 100 mg. minced rabbit kidney in 2 cc. rabbit serum and 2 cc. Tyrode solution. The same technique was followed as for the cultivation of the virus in testicular tissue. In none of the four experiments did pseudorabies virus multiply.

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From the 5th passage in chick embryo medium the virus was cultivated in both serum-containing (A) and in serum-free (B) media. The medium of Group A was the same as used in the previous culture series; while the medium of Group B, 100–150 mg. of minced chick embryo tissue suspended in 4 cc. Tyrode solution, was the same as used by Li and Rivers (19) for the cultivation of vaccinia virus.

TABLE VII

Cultivation of Pseudorabies Virus in Chick Embryo Tissue

No. of serial culture passage*	Test for virulence		Titer for mice
	Guinea pigs (subcutaneously)	Mice (intraperitoneally)	
Ch. E. I	•†	+	•
" II	+	•	•
" III	+	•	•
" IV	•	+	1:100
" V A	•	+	•
" V B	•	+	•
" VII A	+	•	•
" VII B	+	+	•
" X A	•	+	1:10
" X B	•	+	1:100
" XII A	+	•	•
" XII B	+	•	•
" XIII A	•	+	1:1
" XIII B	•	+	1:100
" XVI B‡	+	•	•
" XIX B	•	+	•

* Started from Transfer XXX in rabbit testicle medium.

† • = not tested; + = virulent.

‡ Series A discontinued.

In the rabbit and guinea pig testicle media a certain amount of serum had been necessary to insure multiplication of pseudorabies virus, but in chick embryo cultures serum was not necessary. The growth of the virus was even better in cultures which did not contain serum (Table VII). The titer of the chick embryo cultures, on the average, was considerably lower than that of the rabbit testicle cultures.

After Culture Series Ch. E. XIII the cultivation in media containing serum was discontinued. In serum-free media the virus was still under cultivation when this paper was completed.

bryo medium, however, is the simplest, as it does not require serum, while the testicle media must contain a certain amount of homologous serum to insure multiplication of the virus. In rabbit kidney tissue, a medium suitable for the cultivation of vaccinia virus, pseudorabies could not be grown.

The pathogenic properties of pseudorabies virus were not altered during the 49 serial culture passages and there were merely quantitative differences between the cultivated virus and rabbit brain passage virus. This was to be expected from the experience of others, since, so far as we know, no filtrable virus has changed its pathogenic properties qualitatively during the course of its cultivation *in vitro.*

SUMMARY

Pseudorabies virus has been cultivated in series in rabbit testicle, guinea pig testicle, and chick embryo media, and its growth requirements have been studied. Intranuclear inclusions, similar to those produced by pseudorabies virus *in vivo*, have been found in rabbit testicle cultures. The virus has not changed its pathogenic properties for rabbits, guinea pigs, or mice during the course of cultivation.

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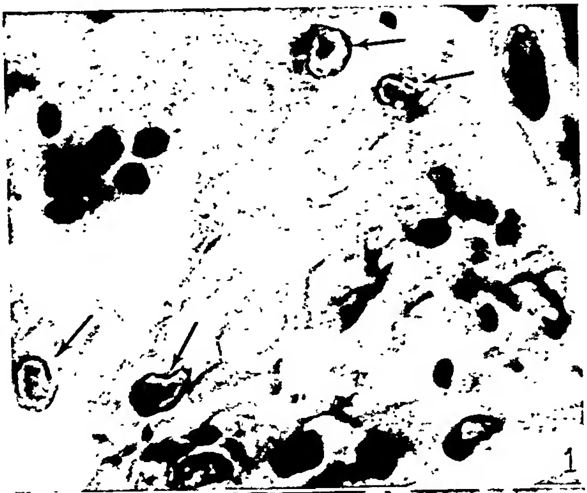
EXPLANATION OF PLATE 40

FIG. 1. Section through a piece of rabbit testicle tissue from a culture incubated for 2 days. Intranuclear inclusions appear in interstitial cells. Hematoxylin-eosin. ×1,033.

FIG. 2. Section through a piece of rabbit testicle tissue from a culture incubated for 4 days. General necrosis. Chromatin has disappeared from the nuclei in the seminiferous tubule (right side of picture). Nuclei of interstitial cells karryorrhectic. Inclusions no longer distinct. Hematoxylin-eosin. ×804.

FIG. 3. Section through a piece of rabbit testicle tissue from uninoculated control media incubated for 4 days. Necrosis much less advanced than in Fig. 2. Nuclei of seminiferous tubule (right side of picture) still contain chromatin. Interstitial cells fairly healthy. Nucleoli still visible. Hematoxylin-eosin. ×804.



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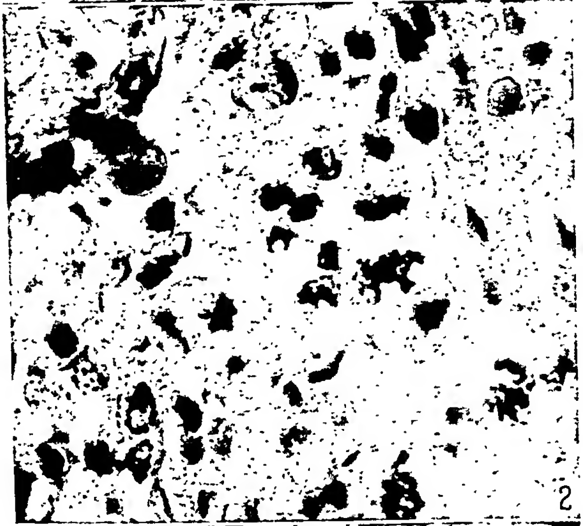

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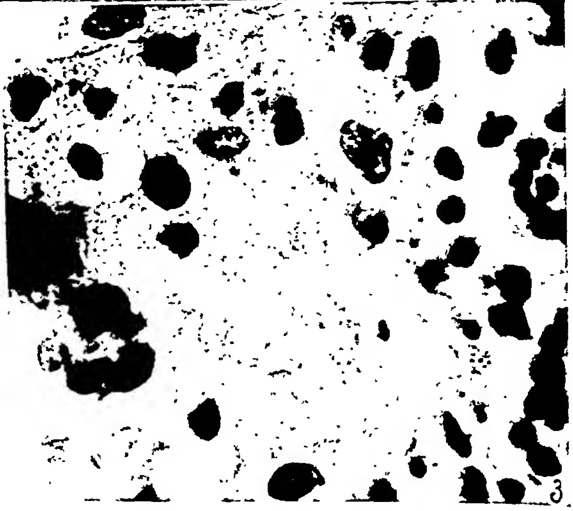

3

(Traub: Cultivation of pseudorabies virus)

THE ACTION OF TYPE-SPECIFIC HEMOPHILUS INFLUENZAE ANTISERUM

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(Received for publication, August 10, 1933)

In a previous study (1) of type-specific strains of *Hemophilus influenzae*, it was observed that the organisms of all the strains isolated from cases of "influenzal meningitis" were encapsulated, formed colonies which have been described as smooth, and produced a soluble specific substance which precipitated the homologous antiserum. At the time the above report was made, organisms from nine patients not closely associated had been studied, and all of the strains were similar and belonged to the group which has been designated as Type b. This suggested that most, or at least the majority of, meningitis strains were of the same serological type.

In 1922, Dr. T. M. Rivers concentrated broth cultures of two meningitis strains and then removed from the concentrates the alcohol-precipitable fractions. 9 years later, these fractions were given to the author to be tested immunologically. Solutions of the fractions from both strains were made, and in each solution a heavy precipitate was formed when Type b antiserum only was added. This indicated, therefore, that the two strains studied by Dr. Rivers were also of the Type b group.

It was mentioned previously (1) that Wollstein (2), Povitsky and Denny (3), Rivers and Kohn (4), and others, found a considerable degree of immunological relationship among the strains isolated from cases of meningitis. There may be some question, however, whether the relationship which they noted was that based on the possession of the common soluble specific substance which we have described. Many of the strains had been isolated a number of months before they were immunologically tested, and as organisms of the S form usually tend to change to those of the R form with great rapidity, it is probable that some of their cultures contained only R forms. Furthermore, the agglutination reactions studied by these observers were carried out at high temperatures (45°C. or higher), under which conditions type-specific reactions are at least partially masked.

Ward and Wright (5), and Ward and Fothergill (6), have also reported that they have observed a close immunological relationship between meningitis strains.

Our observation that the majority of meningitis strains are type-specific and of the same type has been further substantiated in the continuation of this study. Up to the present, 41 strains of Pfeiffer bacilli obtained from patients suffering from lesions of the central nervous system have been examined, and all but four have been of Type b.[1] Three of these exceptions will be discussed later, and in the fourth instance the bacterium isolated was not of the species *Hemophilus influenzae*, but of the species *Hemophilus parainfluenzae*. This organism was isolated by Dr. J. D. Trask from the spinal fluid of a child who had a brain abscess.

The discovery that the organisms present in influenzal meningitis are usually type-specific, and that most of them are immunologically identical, suggested that a highly immune horse serum might have therapeutic value. Earlier attempts by other investigators to treat influenzal meningitis with immune serum were not very successful.

Wollstein (7), and also Rivers (8), did not find *H. influenzae* antiserum very effective in treating cases of influenzal meningitis in children, although Wollstein (9) did find that immune serum was effective in treating monkeys suffering from meningitis experimentally produced.

Reports of patients treated with the Wollstein serum have been made by Torrey (10), Packard (11), and Dunn (12). Torrey and Packard each treated one case, and both, children 11 years old, recovered. Dunn treated eleven cases and all died. He considered that in none of these was the serum treatment started early enough to expect the best results.

In 1921, Neal (13) reported that five patients had been treated with anti-influenzal serum by the Meningitis Division of the New York Department of Health. One patient who was treated with a number of intraspinal injections of vaccine and a few injections of antiserum recovered. In 1933, Neal (14) stated that among 90 cases of influenzal meningitis under their care, there had been three recoveries. These recoveries she did not definitely ascribe to the use of influenza antiserum, but she mentions that she was impressed with the marked, though temporary, improvement in clinical symptoms which followed the use of the serum.

Notwithstanding the earlier failures and the fact that the anatomical conditions in influenzal meningitis are such as to render the local application of any specific treatment difficult, if not impossible, it was

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[1] Certain of these strains were received from Dr. Martha Wollstein, Babies Hospital, New York, Dr. Ann G. Kuttner, Pediatric Clinic of Johns Hopkins Hospital, and the physicians listed in footnote 2, to all of whom the author is greatly indebted.

decided that in the light of the newer knowledge concerning these organisms, a further study of this problem should be made.

Production of Immune Serum in a Horse

In February, 1931, the immunization of a horse with Type b organisms was started.

The antigen used for the earlier as well as the later injections has been prepared by growing Type b meningitis strains for 18 hours on Levinthal agar (1) made with horse blood, and washing the bacteria from each plate with 6 cc. of 0.4 per cent formalin in 0.85 per cent NaCl solution. A fresh lot of vaccine has been prepared for each series of inoculations. Up to the present, twenty-seven different strains have been used. For the first vaccine, five strains were employed; then, as new strains were obtained, the old strains were discarded and the new ones substituted. Great precautions have been taken to employ only pure S cultures in the preparation of the vaccine.

The horse has been immunized by giving a series of five daily intravenous injections of the vaccine followed by a rest period of 9 days before beginning a new series. As marked reactions occurred following the injections, very small inocula of vaccine had to be employed. For the first series, two inoculations of 0.05 cc. of vaccine were made, then the amount was slightly increased for each of the remaining three injections of this series. For the first inoculation of each of the following series of injections, the amount given was a trifle greater than that of the first inoculum of the preceding series, and the dosage was then increased daily. This method of increase was continued until a large bleeding was made, after which the horse was allowed to rest for several weeks. In renewing the process of immunization following the rest, very small inocula were again at first employed, but the size of the dose was increased more rapidly than before.

After each injection, the horse has had a febrile reaction which has reached its maximum in 4 to 7 hours. This reaction has usually been greatest on the 1st and 2nd days of each series of injections. Besides the febrile reaction, the horse has at times had very labored breathing, increased heart rate, weakness of the legs, and marked injection of the blood vessels of the sclerae.

In order to follow the progress of the immunization, a small amount of blood was withdrawn from the horse before the first inoculation of each series, and the serum was tested for its content in type-specific antibodies by means of precipitation and agglutination reactions. For $3\frac{1}{2}$ months, a gradual increase in the precipitating and agglutinating power of the serum occurred. Since then, there has been no apparent change. Precipitation occurs when the Type b purified capsular polysaccharide in dilutions up to one part in one million is added to the serum. Type b bacilli are agglutinated with disc formation in dilutions of the serum up to 1–80, and with granular clumping in higher dilutions up to 1–320.

The serological reactions are carried out at 37°C. for 2 hours, and the tubes are then kept in the ice box overnight before the final readings are made. If the tests are made at temperatures higher than 37°C., disc formation becomes less striking and agglutination becomes largely of the granular type.

The precipitation reaction appears to be the more specific test, and after 2 years of immunization the serum contains no precipitating antibodies for soluble specific substances derived from influenza bacilli other than Type b. On the other hand, after the horse had been under immunization for about a year, it was found that the serum had acquired the ability to agglutinate influenza bacilli of other types. This non-type-specific agglutination is probably due to the presence in the serum of an antibody against some fraction of the cell other than the soluble specific substance. Preliminary experiments indicate that this fraction is carbohydrate in composition, that it is present to some degree in all influenza bacilli, and that it is probably analogous to the C substance of Streptococcus (15) and Pneumococcus (16).

It has thus been possible to produce in a horse an immune serum which is highly specific for Type b influenza bacilli, as shown by precipitation tests. It now seemed important to determine, if possible, whether the serum would exert specific effects in the infected animal as well as in the test-tube. In case the serum were found to have therapeutic value, it also seemed important to learn whether quantitative differences in the relative value of the several lots of serum could be demonstrated.

Consequently, a series of experiments has been made to determine the action of the serum in infected animals.

The Effect of Immune Serum on Mice Infected with Hemophilus influenzae

Since the susceptibility to infection with *H. influenzae* varies markedly in individual mice, and since large inocula are necessary to produce lethal results, it seemed probable that a method of testing based on protective power for mice would not be suitable for determining the immunological value of this serum. Attempts were made, herefore, to evaluate the action of the serum in preventing or inhibiting invasion of the blood following intraperitoneal infections. The bacteria and serum were injected simultaneously, and at various intervals cultures were made of blood from the end of the tail, and the number of bacteria in the blood determined. In a few of the experiments, cultures were also made at the same time from the peritoneal cavity. The results of one of these experiments are given in Table I.

TABLE I

Protection of Mice against Hemophilus influenzae

Culture inoculum	Antiserum Lot 2	Blood cultures—hrs. after inoculation								
		Peripheral								Heart
		1	2	3	4	5	6	7	24	48
cc.	*cc.*									
0.5		++	+++	++++	++++	D				
0.5		++	+++	++	++++	++	+	+	+++	—
1.0		+++	++++	++++	++++	D				
1.0		++++	++++	++++	++++	D				
0.5	0.2	—	—	—	(2)	—	(1)	—	—	—
0.5	0.2	—	—	—	—	—	—	—	—	—
1.0	0.2	—	(2)	(5)	+	++	++	++	(1)	D 31 hrs. (2)
1.0	0.2	(2)	(1)	(6)	(5)	(6)	(2)	—	+	—

—, +, ++, +++, ++++ = none, few, moderate number, many, very many colonies that grew from 1 loopful of blood on Levinthal agar.

Numerals within parentheses indicate the actual number of colonies which grew from 1 loopful of blood.

D = death of animal.

The surviving mice were killed after 48 hours.

It is seen that in the serum-treated mice the invasion of the circulating blood by the bacteria was either prevented or limited, and that in those mice in which invasion occurred the severity of the septicemia was slight, and that three out of four mice survived. The fourth mouse lived for 31 hours, at no time did it have a severe septicemia, and from the heart's blood culture made at autopsy only two colonies developed. In other experiments, however, a few of the treated mice have died as rapidly as the untreated ones. Yet in all experiments the serum-treated mice which succumbed have never had more than a mild septicemia, and in some instances the blood cultures have been sterile. Moreover, in the treated mice which have died the number of bacteria in the peritoneal cavity has been markedly reduced, and the bacteria have been swollen and globoid, and undergoing phagocytosis.

On the other hand, in the experiment recorded in Table I, within 1 hour the untreated mice had moderate to severe septicemia, and at the end of 5 hours three of the four mice were dead. The remaining mouse had a marked septicemia at the end of 24 hours, but the heart's blood culture was sterile when the mouse was killed at the end of 48 hours. In all of the experiments, blood cultures from the untreated mice which succumbed were positive, and cultures made from the peritoneal fluid of these animals showed heavy growths.

All of the animals, treated as well as untreated, have exhibited certain toxic symptoms such as diarrhea and conjunctivitis, yet these symptoms have disappeared more rapidly in the treated animals than in the untreated animals which recovered.

Control experiments have shown that the action of the horse serum is type-specific, since no effect of the serum could be demonstrated in mice infected with Type a or R strains. Moreover, it has been shown that the administration of normal horse serum has no effect on mice infected with Type b strains.

While it was possible to show that the immune horse serum has a specific effect on mice infected with Type b influenza bacilli, it was difficult to estimate this effect quantitatively. Consequently, it was decided to attempt to determine the effect of the serum on larger animals, and since the rabbit, in proportion to its weight, is apparently the least resistant of the ordinary laboratory animals to infection with *H. influenzae*, this animal was chosen for the experiments.

The Effect of Immune Serum on Rabbits Inoculated Intravenously with Hemophilus influenzae

Rabbits were inoculated intravenously with given amounts of culture, and after 30 minutes to an hour blood cultures were made on plates. Definite amounts of immune serum were then injected intravenously. Shortly after the serum treatment, and then at hourly

treated rabbits, the degree of bacteremia increased in the rabbits which were not treated, or which were treated with normal serum.

One of these rabbits died at the end of 5 hours, and another within 24 hours. Both had many organisms in their blood at the time of death. The third rabbit had a massive septicemia at the end of 24 hours, but at the end of 48 hours only a few organisms grew from the blood. In this animal the blood cultures continued positive for several days. It then developed another infection and was killed. The rabbit which served as the serum control showed no abnormal reactions.

A number of experiments similar in plan to the one just described were made, and the results were similar. Furthermore, similar results were observed when the serum was administered before, or simultaneously with, the culture. In one experiment, however, in which the dosage of culture employed was larger, all the rabbits died. Even under these circumstances, however, there occurred a marked reduction in the number of bacteria in the blood of the animals treated with immune serum, as compared with the number of bacteria in the blood of the control animals. It has been found, as the immunization of the horse has progressed, that the amounts of serum necessary to bring about sterilization of the blood have become smaller.

The immune horse serum, therefore, possesses the power of sterilizing the blood of rabbits infected with homologous organisms, unless the initial inoculum of culture is so great that the animal is completely overcome by the toxicity of the injected bacteria. On the other hand, the serum apparently does not prevent the toxic symptoms which follow the inoculation of animals with *H. influenzae*, since these symptoms occur in the treated as well as in the untreated animals.

These symptoms, which have been described by others, are rapid and labored breathing, loss of muscle tonus, increased peristalsis, refusal to eat, and increased secretion from the conjunctivae. Another feature, apparently not previously mentioned, is injection, and at times hemorrhages, of the scleral vessels. This reaction begins about 1 hour after inoculation, and usually persists for about 24 hours. It apparently corresponds to the injection of the blood vessels and hemorrhages in the internal organs which are seen at autopsy in rabbits after inoculations of *H. influenzae*. A further toxic manifestation which occurs shortly after infection is a change of temperature, which may be either lowered or elevated. In the rabbits which survive, there is a rapid elevation of the temperature, which usually returns to normal in less than 48 hours, and the latter occurs even though the bacteremia may still be present.

TABLE II

Protection of Rabbits against Hemophilus influenzae

Rabbit No.	Culture	Serum	Blood cultures—hrs. after inoculation of culture									
			½	1	2	3	4	5	6	7	24	48
	cc.	*cc.*										
1	0.5	Immune* 0.5	+++ Inoculation of serum	(5)	–	–	–	–	–	–	(6)	–
2	0.5	1.0	+++	+	–	–	–	–	–	–	D–	
3	0.5	2.0	+++	–	–	–	–	–	–	–	–	–
4	0.5	Normal 1.0	+++	+++	+++	++++	++++	D++++				
5	0.5	2.0	+++	+++	+++	++++	++++	++++	++++	++++	++++	+
6	0.5	–	+++	+++	+++	++++	++++	++++	++++	++++	D++++	
7	–	Immune 2.0										

–, +, ++, +++, ++++ = none, few, moderate number, many, very many colonies which grew from 1 loopful of blood.

Numerals within parentheses indicate the actual number of colonies which grew from 1 loopful of blood.

D = death.

* Immune serum from Lot 5.

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1	0.5	Immune* 0.5	+++	Inoculation of serum	(5)	–	–	–	–	–	–	(6)	–
2	0.5	1.0	+++		+	–	–	–	–	–	–	D–	
3	0.5	2.0	+++		–	–	–	–	–	–	–	–	–
4	0.5	Normal 1.0	+++		+++	+++	++++	++++	D++++				
5	0.5	2.0	+++		+++	+++	++++	++++	++++	++++	++++	++++	+
6	0.5	–	+++		+++	+++	++++	++++	++++	++++	++++	D++++	
7	–	Immune 2.0											

–, +, ++, +++, ++++ = none, few, moderate number, many, very many colonies which grew from 1 loopful of blood.

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Although the administration of serum does not prevent the
rence of these toxic symptoms, it apparently does shorten thei
tion. This is probably associated with the inhibition of gro
the bacteria by the serum.

The Action of Immune Serum in Preventing the Lesions Induc Intracutaneous Injections of Hemophilus influenzae

The first description of skin lesions induced by the injection of Pfeiffer
was made in 1894 by Kruse (17). He inoculated large doses of bacteria su
taneously into rabbits. The resulting localized lesions were edematous and hy
emic, yet cultures made from them after 24 hours were sterile. After a few
the inflammation subsided, but a hard mass, potato-like in consistency, persi
for a number of days.

In a previous paper (1), the author has described the lesions resulting from
intracutaneous injection of cultures of S and R influenza bacilli in rabbits, and
drawn attention to the employment of this technique for determining the relati
virulence of different strains.

Although previous observations had been made of the skin lesion
induced by *H. influenzae*, it seemed important, before studying the
action of the immune serum on the development of these lesions, to
make a more detailed study of the effects produced by the injection
of different amounts of various strains, both living and dead.

It was found that when a massive inoculation, consisting of the living S organisms concentrated from 1 cc. of a broth culture, is given intradermally, the localized lesion may reach 20 to 40 mm. or more in diameter. The lesion is markedly edematous, at first bright red, then purple-red in color. Later, the center becomes necrotic and a scab forms which covers a thick fibrinopurulent exudate—rarely is there any discharge. The reaction usually begins to decrease after 48 hours, but some inflammatory reaction remains for 5 to 7 days. As the inflammation subsides, a large hard palpable area of induration becomes evident. This may persist for as long as 20 days.

When massive inocula of R organisms are injected, lesions of almost equal severity develop. Furthermore, similar reactions develop if large amounts of dead organisms, either S or R, are given. However, the intensity of the lesions induced by the different forms bears a very definite relationship to the amount of culture injected. When small amounts are given, it is observed that the living S forms have a much greater capacity to induce lesions than do the dead S, or living or dead R forms. Of the three latter forms, there seems to be no significant difference in the reactions which they induce.

To illustrate the capacity of these forms of bacteria to induce lesions, Table III is given, in which is indicated the relative severity of the reactions usually observed.

Since dead forms of these bacteria are capable of inducing lesions, it is obvious that at least some of the effects of intracutaneous inoculations are due to preformed substances present in the bacterial cells. Further, since the lesions induced by living R forms are no more severe than those induced by the dead R forms, it seems probable that in the case of the R forms the lesions are entirely due to preformed substances, the living R forms being quickly killed after the inoculation.

TABLE III

Comparison of Skin Lesions Induced by S and R Forms of Hemophilus influenzae

Amount of culture in 0.2 cc. volume	Qualitative differences in the lesions induced by		
	Living S	Living R	Dead S or R
cc.			
1.0	++++	+++	+++
0.1	+++	+	+
0.01	++±	±	±
0.001	++	−	−
0.0001	+	−	−
0.00001	±	−	−

−, ±, +, ++, +++, ++++ = no reaction, very mild, mild, moderate, severe, and very severe reaction.

In the case of the S forms, however, it is noted that a much smaller dosage of living than of dead bacteria will induce an evident lesion, and in this instance it seems that living S forms are able to multiply and thus produce sufficient irritating substance to give rise to reactions.

Preliminary experiments showed that if a certain amount of immune serum were added to massive doses of the culture, some effect on the extent of the lesion was observable, but that if smaller doses of culture were employed the inhibiting effect of the immune serum was much greater, and that with still smaller doses the production of a lesion might be completely prevented. It was found that the actual amount of serum injected made little difference in the severity of the lesions; if the dose of culture was not too great, small amounts of serum within certain limits were as effective as large amounts. Furthermore, it was observed that when the dose of culture was so large as to produce lesions in spite of the admixture with serum, the lesions were very similar in size to those induced by like numbers of heat-killed bacteria given alone. Hence, it appeared that the serum could only inhibit the action of a definite number of bacteria, and that the action of the serum consisted in preventing the growth of the bacteria rather than in neutralizing the toxic or irritating substances.

It was therefore evident that in employing skin inoculations to make quantitative tests of the inhibitory action of different lots of serum, it would be necessary to determine with considerable care the optimal dosage of culture to be employed in the tests. A series of observations, therefore, was made, employing various doses of culture alone, and also the same amounts of culture mixed with varying amounts of immune serum.

The culture employed in the tests was Strain 225S, isolated from a patient suffering from meningitis. The strain was kept under optimal conditions to prevent the development of R variants. The technique employed in cultivating and studying the characteristics of the culture was the same as that described previously (1). For the tests, 24 hour broth cultures were employed, and physiological salt solution was used as a diluent rather than broth, as the whole broth alone sometimes causes slight reactions. To prevent injury to the bacterial cells taking place in the salt solution, the injections were made as quickly as possible after diluting the culture. In all cases, 0.1 cc. of the culture or culture dilution was mixed with 0.1 cc. of salt solution, or with 0.1 cc. of whole or diluted serum, the amount of fluid injected in all cases being 0.2 cc.

On the day preceding the tests, the hair was removed from the flanks of the rabbits with electric clippers. Gray rabbits with thick white skins have been found most suitable for the tests.

It was found that when the largest inoculum of culture (0.1 cc.) was injected alone, there was induced a hyperemic edematous lesion 20 to 30 mm. in diameter, with a small central area of necrosis. This inflammatory reaction lasted for 4 to 6 days, and one large palpable area of induration, or several smaller areas, persisted for about 10 days longer. Smaller amounts of the culture induced lesions of less severity and with less induration, but the lesions did not differ markedly in surface area unless the inocula were less than 0.0001 cc. of culture. Amounts less than 0.0001 cc. sometimes induced lesions as large as 15 mm. in diameter, but these rapidly diminished and rarely exceeded 10 mm. at the end of 72 hours, and no palpable areas of induration persisted.

When the serum was injected together with the culture, it was found that 0.001 cc. of culture was the largest dose that could be completely neutralized by the addition of the immune serum, no matter how large an amount of serum was employed. In other words, if doses larger than 0.001 cc. of culture are used, there is apparently sufficient preformed substance present to induce lesions, even though the growth of the bacteria may be completely inhibited. It therefore became obvious that 0.001 cc. was the largest dose of culture which could be used for carrying out a series of tests with different sera. And since this amount was at least ten times greater than an amount (0.0001 cc.) which could produce a persistent lesion, it was decided that 0.001 cc. of culture would be the most satisfactory dose to employ. It was arbitrarily decided that a reaction was to be considered negative if at no time it exceeded 10 mm. in diameter and had completely disappeared within 72 hours.

When the horse was bled at the different intervals, tests were made with various dilutions of the serum, employing 0.001 cc. as a constant standard dose of culture. Control tests were also made with mixtures of culture and normal serum, and with the culture alone. The results of three of these tests are given in Table IV.

The lesions are described by means of linear measurements, but this method of expressing the difference in the lesions is inadequate, as it does not indicate differences in degree of hyperemia and edema.

It will be seen that whereas the administration of normal serum had no effect in reducing the size of the lesions resulting from the intradermal injection of 0.001 cc. of the standard culture, the addition of immune serum had a marked effect on the lesions. Moreover, distinct differences in the effects produced by sera from various bleedings were evident. Employing the standards mentioned above, it is seen that while 1/200 cc. of serum of Lot 1 was necessary to render the skin reaction negative, only 1/600 cc. of serum of Lot 2 was required, and 1/800 cc., or possibly less, of Lot 3.

It is obvious, therefore, that by means of this technique the protective action of immune serum may be demonstrated, and, moreover, that quantitative differences in the relative protective action of different lots of serum may be detected. Lot 1 serum was obtained 4 months after the immunization of the horse was undertaken, Lot 2 serum 3 months later, and Lot 3, 3 months after Lot 2. The results indicate, therefore, that with the continued immunization there occurred a progressive increase in the power of the serum to prevent the occurrence of skin lesions following the injection of a definite number of Type b influenza bacilli.

TABLE IV

Influence of Immune Serum in Preventing Lesions in the Skin of Rabbits

Rabbit No.	Readings	Serum	Area of lesion induced by									
			Serum plus 0.001 cc. of culture							Culture alone		
			1/10 cc.	1/100 cc.	1/200 cc.	1/400 cc.	1/600 cc.	1/800 cc.	1/1000 cc.	0.001 cc.	0.0001 cc.	0.00001 cc.
	hrs.		*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
1	24	Immune		10x10	4x7	14x11	13x12			25x22	18x16	
	72	Lot 1		—	—	10x10	12x12			22x21	10x10	
2	24	Lot 2		8x8	9x9	7x7	8x8	10x12		18x16	10x11	
	72			—	—	—	—	8x10		19x17	10x10	
3	24	Lot 3	—	3x3	—	—	2x2	7x7	10x11	*	15x15	12x12
	72		—	—	—	—	—	—	—		11x12	7x8
	24	Normal	20x15	15x20	15x13							
	72		20x15	15x15	13x11							

Total volume of each inoculum = 0.2 cc.

The sera alone caused no reactions.

Protective titre of immune serum: Lot 1 = 1–200; Lot 2 = 1–600; Lot 3 = 1–800+.

* Material was lost.

The immune serum was also tested with other Type b strains, and the results were the same as when the standard Strain 225S was employed. That the action of the serum was type-specific was demonstrated by observing the lesions induced by the injection of mixtures of serum and living and heat-killed organisms of the other types and R forms. Under these conditions, no preventive action of the serum could be detected. However, the serum used in these cross-protection tests was from the second bleeding, which was made before the appearance of the antibody which seems to be similar to the C antibody, and it is not known what influence this antibody might have on heterologous type skin infections.

The Action of Immune Serum in Patients Suffering from Meningitis

The experimental studies have indicated that the serum of a horse immunized against Type b *H. influenzae* exerts a specific action on these organisms, not only in the test-tube, but also in infected animals. It was decided to study the therapeutic action of this serum in a small series of patients suffering from meningitis due to *H. influenzae*, Type b. As cases of this disease occur mostly in young children and have not been available in this hospital, the cooperation of certain physicians likely to meet with cases of this disease has been enlisted. The writer desires here to express thanks to the physicians who have kindly supplied the histories of the patients.[2]

Eighteen patients have now been treated by means of intrathecal injections of immune horse serum supplied by us. Owing to difficulties in making prompt diagnosis of the bacteria concerned, and lack of opportunities for careful study of the cultures, certain of the cases cannot justifiably be included in discussing the action of the serum. In three cases, the influenza bacilli isolated proved to be not of the Type b form. In one of these cases, the organisms were found to be of Type a, in another of Type f, and in the third case the organisms which grew from the spinal fluid obtained on four occasions early, as well as late in the disease, produced only R colonies. Inasmuch as the serum is considered to be type-specific, no action was expected in patients

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suffering from infections due to heterologous organisms. Nevertheless, in one of these cases, following treatment with the immune serum, the patient apparently recovered from an infection with *H. influenzae* of a heterologous type.

This case, W. B., treated at the Jersey City Hospital, was not typically one of primary meningitis in a child, but was a case of secondary meningitis following traumatic fracture of the skull and laceration of the face occurring in a man 51 years of age. 6 days after the injury he developed signs of meningitis, and 4 days later cultures from the cloudy spinal fluid revealed the presence of *H. influenzae* Type a. The physician was anxious to employ treatment with immune serum, and intraspinal injections of 20 cc. each were made once or twice daily over a period of 19 days. 7 days after the treatment was begun, cultures from the spinal fluid became sterile and remained so. During the period of treatment marked improvement in the patient's physical and mental condition occurred, but about 3 weeks after the treatment was stopped his condition again became worse and he died 1 week later. At autopsy, there was no evidence of meningitis, but a brain abscess was found, cultures from which showed the presence of streptococci and staphylococci, but no influenza bacilli.

Whether or not the serum had any effect in this case is uncertain. Influenza bacillus meningitis rarely occurs in adults, and it is less frequently fatal than in children. Its occurrence in adults has been reviewed by Wollstein (18), Rivers (19), Bloom (20), and others.

The other two cases of this group, the one in which Type f organisms were found, and the one in which the organisms were all of the R form, ended fatally, and there was little evidence that the administration of the serum had any effect on the course of the disease.

There were two further cases in which a thorough study was not made of the infecting organisms. The determination of type was made solely on the agglutination reaction in the immune horse serum, and, in the light of further experience, the conclusion that the organisms were of Type b must be considered doubtful.

There remain thirteen cases in which the infecting organisms were shown conclusively to be of Type b. Unfortunately these patients were treated under widely varying conditions, and by different methods. The ages of these patients ranged between 2 months and 7 years, nine of them being 3 years, or younger. Among these thirteen cases, there was only one in which recovery occurred.

Case I.—This patient, M. P., 2 years of age, was admitted to the Jewish Hospital of Brooklyn on Apr. 24, 1933. There had been a discharge from the ear for 4 weeks. For 3 days the child had had signs of muscular incoordination, as he was reported to have fallen several times. The night before admission he vomited and had a chill, which was followed by a rise in temperature. The following morning he seemed well, but in the evening his temperature again rose and he appeared very limp, drowsy, and listless. The next day he was admitted to the hospital. A diagnosis of meningitis was made, and he was treated with meningococcus antiserum. On the 2nd day, the organisms which grew in the culture from the spinal fluid, and also in the culture from the blood, were identified as influenza bacilli, and treatment with specific immune serum was started. On this day he was given two intravenous and two intralumbar injections of immune serum, all of 20 cc. each. On each of the 4 succeeding days he was given two intralumbar treatments of 20 cc. of immune serum to which 2 cc. of fresh human serum had been added, on each of the next 2 days one treatment of 10 cc. of immune serum plus 2 cc. of fresh serum, and on the following day one intralumbar injection of 10 cc. of immune serum alone. On the 2nd day of specific treatment he was also given an intravenous injection of 10 cc. of immune serum. After the first 24 hours of treatment, cultures from the spinal fluid were sterile. This was after two positive cultures had been obtained from the spinal fluid, and before any fresh serum had been injected. The blood culture was positive on admission, and no further cultures were made from the blood until 1 week later, at which time no growth occurred. During the period of specific treatment, the number of leucocytes in the spinal fluid gradually decreased from 8900 to 460 per c.mm., and 10 days later the cell count was 5. Clinically, the child progressively improved, the meningitic symptoms gradually disappearing. The temperature slowly dropped, and after May 2 remained normal. The patient, apparently well, was discharged May 13.

Shortly after admission to the hospital, nose and throat cultures were made from which Type b influenza bacilli were grown. 10 days after discharge, cultures were again made from the nose and throat, and from the nose culture Type b organisms were again recovered. The latest cultures were made on June 29 from excised tonsils and adenoids, and on the plates a few colonies of Type b organisms and many colonies of R forms developed.

In two of the thirteen cases, the spinal fluid became sterile following the administration of immune serum, and remained so for periods of 7 to 14 days, but in both instances the organisms reappeared and death ensued. Brief reports of these cases follow.

Case II.—R. S., 2¾ years old, was admitted to the Babies Hospital, New York City, Mar. 8, 1932. A diagnosis of influenzal meningitis was made, and the child was treated intraspinally with immune serum to which fresh human serum was added. For 4 days the number of influenza bacilli in the spinal fluid diminished, on the 5th day no growth was obtained, and for the next 6 days the cultures were sterile. During this time the serum treatment was continued and the child improved clinically. However, the child then developed otitis media, streptococci were recovered from the discharge, and influenza bacilli again appeared in cultures from the spinal fluid. After this, cultures from the spinal fluid were again sterile for several days, but the influenza bacilli reappeared once more and were present in all subsequent cultures until death, which occurred on Apr. 9.

Case III.—J. B., 3½ years old, was admitted to Beth-El Hospital, Brooklyn, Dec. 5, 1932. The child had been ill for 4 days, and for 3 days meningitic symptoms had been present. On admission, a lumbar puncture was made, a cloudy fluid was withdrawn, and the child was treated with meningococcus antiserum. But, on examination of a smear of the spinal fluid, a diagnosis of influenzal meningitis was made, and later that day the child was treated with specific immune serum. For the next 7 days the child was given two daily intralumbar injections of serum, of approximately 20 cc. each. Positive cultures of *H. influenzae* were obtained from the spinal fluid daily for 4 days after the beginning of treatment, then the cultures became negative (Dec. 10) and remained so until Dec. 24. On Dec. 12 the spinal fluid was clear, the cell count 100, and the sugar content normal, but as the temperature continued elevated a block was suspected. A cisternal puncture was made and fluid of the same appearance as that obtained from the lumbar tap was withdrawn. This fluid was replaced by serum. Cultures made from this fluid were sterile. From Dec. 12 to 22 the child was given one daily intralumbar injection of immune serum, 20 cc. on each occasion. During this time the child showed improvement and ate and slept well. The strabismus which had been present disappeared, but double otitis media developed and both drums were punctured on Dec. 18. On Dec. 24 the child's condition became much worse. Bilateral mastoiditis developed, and it was found that the spinal fluid had again become positive for influenza bacilli. 4 days later both mastoids were opened. The serum treatment was continued after the operation, but the child progressively grew worse and died Jan. 1, 1933. No fresh serum was used in the treatment of this case.

In another case, there was a reduction in the number of bacteria in the spinal fluid following intraspinal treatment, and on one occasion no organisms were recovered either from slant or broth cultures.

Case IV.—R. F., 5 years of age. The onset of this child's illness was very sudden, and she was admitted to the New Haven Hospital Nov. 25, 1931, on the 1st day of illness. A diagnosis of meningitis was made, and she was given an immediate intraspinal treatment with meningococcus antiserum. An examination of a smear of the spinal fluid, however, showed the presence of Gram-negative bacilli, and treatment with *H. influenzae* antiserum was begun. The next day the child was given two intraspinal treatments, the 5 succeeding days one intraspinal or intracisternal treatment daily, and the 8th day two intraspinal treatments. No definite clinical improvement was noted, however, and the serum treatment was discontinued. The child died on the 26th day. On admission, the blood culture was positive, but on the 3rd day after treatment, and also on three subsequent occasions, it was negative, the last negative culture being obtained 10 days after the cessation of serum treatment. However, 4 days later a positive blood culture was again obtained, and death occurred the 4th day following. The spinal fluid cultures following the administration of the serum showed a reduction in the number of bacteria present, and on one occasion, after three intraspinal treatments, the cultures were sterile. But on all subsequent occasions the spinal fluid cultures were positive.

In each of three other cases (Cases V, VI, and VII) in which treatment was commenced on the 7th, 5th, and 2nd days of illness, respectively, there occurred a temporary decrease in the number of bacteria in the spinal fluid following the treatment with immune serum. Later, the organisms again became numerous in the spinal fluid and remained so until death. In Cases V and VII, the cultures from the blood, which were positive before treatment was commenced, later became sterile.

In Case V, two positive blood cultures were obtained on 2 successive days. 2 hours after the patient had received 20 cc. of immune serum intraspinally, and 20 cc. intravenously, the cultures from the blood were sterile. Blood cultures were also sterile 2 days later, but on the following day, 24 hours after serum treatment had been discontinued, the blood cultures were again positive. The patient died 2 days later.

In Case VII, 2 days after a total amount of 60 cc. of influenzal antiserum plus 15 cc. of fresh human serum had been given intraspinally, and 120 cc. of influenzal antiserum intravenously, the blood culture was negative. Blood cultures on the following day were also negative. The child lived 2 days longer, during which time the specific treatment was continued, but no reports of further blood cultures were made.

Among the remaining six cases, there occurred no significant changes in the condition of the patients or in the character of the spinal fluid following the administration of immune serum. In two of these only intracisternal treatments were given on account of inability to withdraw fluid by the lumbar route, and in one, two intraspinal injections were made, after which the serum treatment was discontinued because of inability to withdraw spinal fluid by this route.

It is realized that the results obtained in the treatment of these thirteen cases do not indicate that this form of specific therapy, carried out under the given conditions, was of great practical value. It must be borne in mind, however, that influenzal meningitis is a very serious condition, and that without specific treatment almost all of those afflicted die. Moreover among the cases here reported two were complicated by pneumonia and empyema. Six were treated very late in the course of the meningitis, and in certain instances treatment was carried out only over short periods of time. It is possible that in a group of cases treated earlier in the course of the disease with greater intensity, and over prolonged periods, the results might be better.

The most important evidence presented by this study, indicating that the administration of the serum may exert an influence on the course of the disease, is given by the results of cultures from the blood and spinal fluid before and after the administration of serum. In seven of the cases, influenza bacilli grew in cultures from the blood before specific serum treatment was administered. In two of these cases no further reports were obtained on the course of the blood infection. In one case, the patient was suffering also from pneumonia, and the septicemia was uninfluenced by the administration of serum. In the remaining four cases with positive blood cultures, the blood became sterile after treatment with serum; in one instance (Case I) the patient recovered, in another (Case VII) there was no report of a recurrence of blood invasion, while in two cases (Cases IV and V), in which treatment was discontinued, the blood cultures again became positive before death. The total number of cases is small, but it seems not unlikely that, in the four cases mentioned, the administration of immune serum had the effect of at least temporarily sterilizing the blood.

The results of the intrathecal injection of immune serum on the bacteria in the spinal fluid varied, but in certain instances, at least, the serum seemed to have a definite effect. In one case (Case I) the bacteria disappeared from the spinal fluid following treatment, and recovery occurred. In two cases (Cases II and III) the spinal fluid became sterile and remained sterile for 7 and 14 days, respectively. In one case (Case IV) there occurred a reduction in the number of bacteria, and on one occasion the culture was sterile. In three other cases (Cases V, VI, and VII) there occurred a reduction in the number of bacteria, as shown by smears and cultures. In the remaining six cases, no changes in the number of bacteria in the spinal fluid were noted.

Recently, Ward and Fothergill (6), and Ward and Wright (5), have reported concerning the treatment of eight cases of influenzal meningitis with an immune serum produced by immunization of a horse with meningitic strains of *H. influenzae.* In one case recovery occurred, and in five, after treatment, the cultures of the spinal fluid were sterile for periods of from 1 to 14 days. In all these five cases the bacteria later appeared in the cultures and death ensued. In the treatment of these cases fresh human serum was added to the immune serum. The previous experimental studies of these writers had led them to the conclusion that the action of *H. influenzae* antiserum is bactericidal, brought about by the action of antibody and complement upon the antigen. As they had been unable to demonstrate the presence of complement in the spinal fluid of patients suffering from influenzal menigitis, they considered that complement should be added to the immune serum before making intrathecal injections.

In the treatment of the patients reported in the present paper, in certain cases small amounts of fresh serum were mixed with the immune serum, and in other cases the immune serum was given alone. Our data do not permit definite conclusions to be drawn concerning the importance of the addition of fresh serum. In the one case (Case I) which recovered, however, the spinal fluid cultures became sterile after the injection of the immune serum without the addition of fresh serum, and in another instance (Case III) the spinal fluid cultures became sterile and remained so for a period of 14 days following the administration of immune serum alone. In Case IV, in which a reduction in the number of organisms occurred, and in which the fluid was sterile on one occasion, no fresh serum was added to the immune serum. In three other cases in which there resulted temporary sterilization of the spinal fluid or a reduction in the number of bacteria present, fresh serum was added to the immune serum.

SUMMARY

In this communication, further evidence has been given which supports the view that the majority of the strains of *Hemophilus influen-*

zae giving rise to meningitis are of the same serological type. Fo
strains have now been examined, and thirty-seven have been of Type

A horse has been artificially immunized with Type b strains isolat
from the spinal fluid of patients. By precipitation tests with t
capsular carbohydrate, the serum has been shown to be highly typ
specific. For the first 3½ months of immunization, the *type-specifi*
antibody content of the serum increased steadily. Later, in spite o
continued immunization, there occurred no apparent increase.

By means of animal inoculations, it has been shown that the antiserum has an anti-infectious action. If mice, inoculated intraperitoneally with Type b organisms, were also given serum, the bacteria did not invade the blood, or did so to only a limited degree. But the recovery of the treated mice was found to be inconstant. In rabbits infected intravenously and later treated by the same route, the number of bacteria in the blood stream was quickly reduced and sterilization followed. In the experiments it was necessary that the dosage of the culture be not too large, as influenza bacilli contain a substance which, artificially introduced into mice and rabbits, gives rise to marked toxic reactions. This substance is apparently not neutralized by the antiserum. However, it was found that among the surviving animals, those treated with immune serum returned to the normal state more quickly than did the animals not so treated.

The anti-infectious action of the serum has further been demonstrated by a study of its effect on the lesions which follow inoculations of type-specific bacteria into the skin of rabbits. Again it was found that for any effect of the serum to be manifested it is necessary that the dosage of bacteria be limited, since if large numbers of bacteria are introduced into the skin the development of lesions cannot be completely inhibited, no matter how large doses of serum are employed. As the number of living S organisms which cannot be neutralized is roughly equivalent to the number of R or heat-killed bacteria which may produce a lesion, it seems that there is some preformed irritating substance in the bacterial cells which may give rise to lesions, even if the bacteria are killed or inhibited in their growth. In order to demonstrate the protective action of immune serum, therefore, it has been found necessary to employ a dosage of culture so small that if the bacteria are immediately killed, or their growth inhibited, no lesion results. Employing immune serum under these conditions, it has been found that the ability of the serum to prevent the occurrence of skin lesions has progressively increased with continuing immunization of the horse.

A series of eighteen patients suffering from influenzal meningitis has been treated with Type b antiserum. Following the use of serum, recovery occurred in one patient of the series, and in two, although the patients ultimately died, the spinal fluid cultures became sterile and remained so for periods of 7 to 14 days. In four other cases, the spinal fluid cultures showed, temporarily, either no growth of bacteria, or a reduction of their number. Among five patients in whom septicemia was present before treatment, in four the blood cultures, after treatment with serum, became sterile.

The number of patients treated has been small, and the treatments were carried out under widely varying conditions. It is difficult, therefore, to draw conclusions regarding the actual value of this form of therapy, or the best methods of procedure. The clinical results, however, indicate, as do the experimental, that the serum has a definite anti-infectious action. The experience is too limited to permit final conclusions regarding the importance of the addition of fresh (complement-containing) serum to the immune serum. Further experience, under more accurately controlled conditions, may show that the serum has greater practical value in treatment than is shown by the mortality results in this series of cases.

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CONCERNING THE RELATIVE RESPONSE TO BLOOD GAINS AND BLOOD LOSSES; AND HABITUATION TO AN EXCESS OF BLOOD PIGMENT

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The experiments to be described were begun with the aim of determining the amount of blood, as red cells, formed each day in rabbits. It was assumed that if this amount were supplied from without the erythropoietic tissue would cease to work. Robertson (1) had noted that repeated large transfusions resulting in a superabundance of hemoglobin caused a practical disappearance of reticulated red cells from the circulation of rabbits, indicating that the marrow had become less active. When, after a time, the alien blood was suddenly destroyed as result of the formation of immune bodies by the recipient, a profound anemia developed, this fact as well as others showing that the transfused cells had functioned so effectively that the marrow had largely left off erythrocyte production. In some recipients no blood destruction was evident, and these formed no antibodies demonstrable on test. For the purpose of the present work such a state of affairs was essential. A measured small amount of compatible blood was introduced into the circulation each day, with the expectation that the marrow would lessen its erythropoietic activity in proportion as its task was taken over, the amount of strange blood necessary to induce it to cease work being presumably that which it would form each day under normal conditions. The results of the experiments have proved the primary assumption to be incorrect. No indication has been found that marrow activity lessens when small quantities of compatible blood are added to the circulation day after day. On the contrary, the marrow keeps on working and itself contributes to the increase in hemoglobin. Not only this, but when no more blood is introduced from without, and the superabundance of hemoglobin begins to diminish, the marrow becomes abnormally active, producing red cells in such quantity as to maintain the abnormal state of affairs. An habituation of the organism to this state has come about.

There would appear to be no papers in the literature on the consequences of bringing about a superabundance of hemoglobin gradually by experimental means, though there are many describing the consequences of doing this abruptly, and many dealing specifically with the relation between marrow activity and hemoglobin concentration. Boycott and Oakley (2) have recently dealt with the latter theme comprehensively, in a paper appearing since the present work was completed. Interested primarily in the regulation of marrow activity, they undertook to stop it by supplying blood from without; but this they found themselves unable to do. The conditions were drastically altered in their experiments, and their findings differ in many respects from those now to be reported. Their data, considered in parallel with our own, provide numerous enlightening contrasts. They discuss in detail some of the problems of marrow activity and for these, as for not a few other pertinent matters, it has seemed well to refer the reader to their paper rather than to attempt to recapitulate what has been admirably summed up.

The Effects of Gradual Additions to the Blood

A rough, first test was made to find how much blood must be introduced daily into the circulation of rabbits in order to cause the erythropoietic tissue to stop working. As a criterion of stopping work the disappearance of reticulated red cells was looked for; and at the end of the series of transfusions the red marrow of some of the animals was examined for its content of these cells. A group of six normal rabbits were given transfusions of whole compatible blood, 6 days in 7, the daily amounts being $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, 3, and 4 cc. for the respective animals.

An extensive literature shows that the number of reticulocytes in circulation varies with the erythropoietic activity; and dependence is now justifiably placed upon the count as indicative of what is occurring in the red marrow. Boycott and Oakley discuss these points at length and they have succinctly stated the general conclusion of investigators:—" . . . while changes in the proportion of reticulocytes are a good index of changes in marrow activity they are not a directly quantitative measure of it." Robertson (1) found that the marrow of rabbits maintained in plethora by the injection of large amounts of blood showed but few reticulocytes as compared with the normal content.

The rabbits, normal animals of mixed breed from stock, weighed from 1200 to 1550 gm. at the beginning of the transfusions and from 1600 to 2100 gm. at their end. They were kept in separate cages and fed hay daily, with the addition three times a week of a mixture of equal parts of oats and commercial food pellets. Water was available to them at all times.

In this series we followed the method employed by Robertson (1), aspirating the blood directly from the heart into a syringe containing 1 cc. of a 1 per cent solution of sodium citrate in normal saline. Normal compatible donors were employed in rotation, each being discarded after two or three bleedings to the amount necessary for all of the transfusions of a day. The method of Rous and Turner (3) was employed in the tests for compatibility. For 20 days before the transfusions were begun and just prior to each of these latter, reticulocyte counts and determinations of the blood hemoglobin were made. During the preliminary period the amount of hemoglobin did not vary significantly, but in several cases (Charts 1 and 2) the number of reticulocytes increased gradually.

The blood samples for counts and hemoglobin determinations were regularly taken in the morning, before the feeding of the day. Both counts and determinations were done by the same person in all of the experiments. They were made on blood from the vein of an ear rendered hyperemic by contact with a bottle of warm water. For the hemoglobin determinations, 20 c.mm. of blood was mixed with 5 cc. of 0.1 N hydrochloric acid and allowed to stand at least 1 hour. The readings were made by means of a Duboscq colorimeter supplied with the yellow glass matching-disc of Newcomer (4). The findings are expressed in grams per 100 cc. of blood.

A white cell pipette was used for the reticulocyte counts. Blood was drawn up to the 1 division and diluted to 11 (1–10) with the staining mixture employed by Friedlander and Wiedeman (5). Staining took place for 15 minutes when a drop of the mixture was placed on a slide and counting was begun at once of the reticulocytes occurring among a thousand cells in uniform fields.

The eventual reticulocyte determinations on the red marrow were made in two ways. In one a small piece of marrow was smeared on a slide previously prepared by allowing a concentrated alcoholic solution of brilliant cresyl blue to dry upon it, thus leaving a film of the dye. Such preparations could be counterstained with Wright's stain and were useful in examining for other manifestations of bone marrow activity. The second method was to wash out a portion of the marrow by forced injections into it of normal saline through a hypodermic needle after the method of Robertson. The mixture thus obtained was spun and counts were made on the sediment diluted with staining mixture as in the case of the blood samples. The period of transfusion ranged from 30 to 70 days.

In only one of the six recipients did an evident incompatibility develop to mar the findings. In this animal, which received 3 cc. of blood each day with result that the hemoglobin percentage mounted rapidly, there occurred the characteristic phenomenon first described by Robertson; namely, a suddenly developing, pronounced anemia although the transfusions were continued. With the anemia there was associated an appearance of strong isohemagglutinins in the blood.

The circulating hemoglobin diminished from 11 gm. to 4 gm. per ce between the 7th day and the 14th day of transfusion, and then rapidl mounted again, the percentage of reticulocytes rising from 20 to 55 per thousand as repair took place. The animal will not be considere further.

No hemoglobin increase developed in the rabbit receiving ½ cc. of compatible blood per day during more than 1 month of transfusion, and its reticulocytes varied throughout within the limits of the pre-transfusion normal. In each of the other four rabbits, those receiving daily 1, 1½, 2, and 4 cc. of blood respectively, the hemoglobin percentage increased,—gradually in the case of the rabbits receiving the smaller amounts of blood, promptly and greatly in the one injected with 4 cc. In all these instances some depression of marrow activity occurred as evidenced by a drop in the reticulocyte percentage; but it did not fall enduringly below the level at the beginning of the pre-transfusion period, and at autopsy the marrow count of reticulocytes was not significantly different from the normal, great numbers of these cells being present. Charts 1 and 2 illustrate the findings.

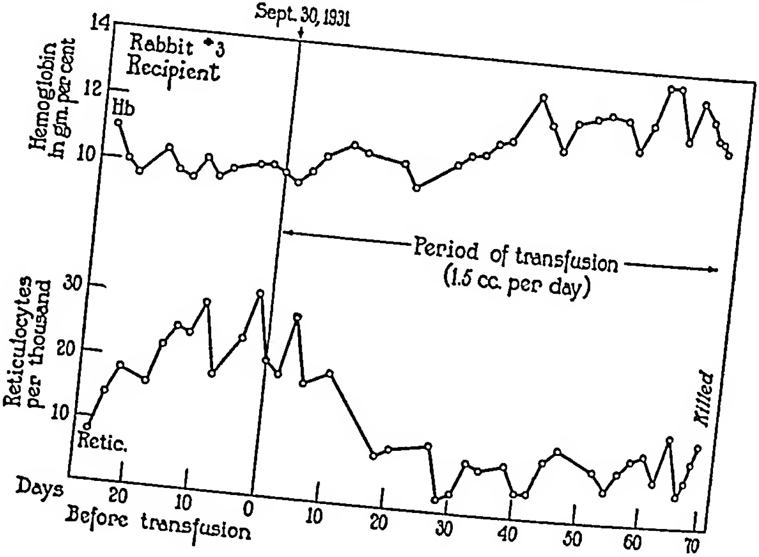

CHART 1

As already stated, the animals serving as recipients were "normals" selected from stock. It seemed possible that their initial amounts of hemoglobin (9 to 10 gm. per cent) might have been near the lower limit for normality, and that the hemoglobin increase, after the smaller transfusions at least, might not have constituted a superabundance of the pigment but have been only a natural betterment consequent upon unusually favorable conditions for blood production. The rabbits were still growing rapidly, which introduced another variable. In the attempt to control conditions more strictly, a new group of animals was studied, adults selected as having large initial quantities of hemoglobin in the circulating blood. Two had 12 and 13 gm. per cent, and these received every day for 35 days without exception $\frac{1}{2}$ cc. of compatible blood; while three other animals with 11, 12, and 12.1 gm. per cent of hemoglobin respectively were given 1 cc. *per diem.* The weights ranged from 1880 to 2280 gm. at the beginning of the transfusions and from 2100 to 2450 gm. at their end. The same technique of transfusion was employed as in the previous experiment.

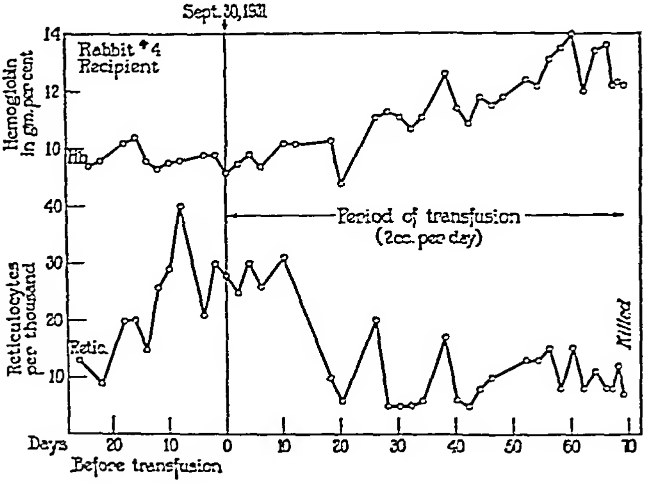

CHART 2

The observations on the two animals first mentioned were marred by intercurrent illness (snuffles) in one case and by the development of antibodies against the alien blood in the other; but these complications did not develop until after a progressive increase in hemoglobin had occurred as result of the transfusions. Despite this increase no drop in the reticulocytes took place. In the rabbits receiving 1 cc. of blood each day for 35 days there were more considerable increases in hemoglobin, to as much as 14 gm. in one case; yet the reticulocyte percentage, though touching zero on a single occasion in one of the animals, underwent no enduring, significant reduction.

The results in this series confirmed the previous observations. Even in rabbits with what appeared to be an abundance of hemoglobin, the introduction from without of 1 cc. of blood each day caused a considerable increase in the amount of the pigment per 100 cc. of blood. Yet reticulated cells continued to circulate in good numbers, whence one might infer that there was no considerable marrow depression. The findings will not be recorded in detail since better controlled and far more convincing evidence was obtained in the next group of animals.

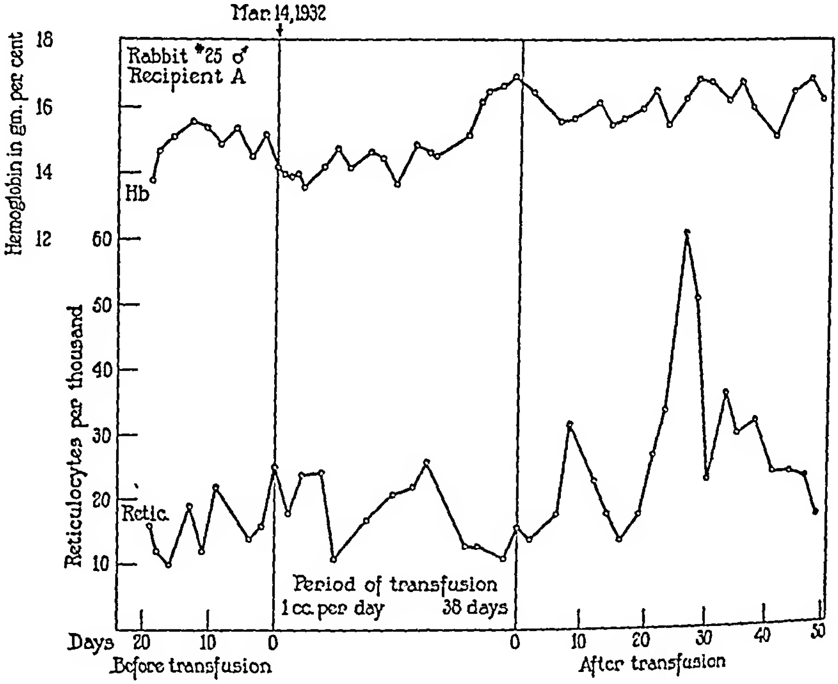

CHART 3

For the purposes of this experiment, adult rabbits were selected, both as recipients and as donors, that had notably great amounts of circulating hemoglobin,—far beyond the average "normal" quantity. A single donor was provided for each recipient in order to narrow the chances that the introduced blood might have a hidden incompatibility;

and frequent tests were made to be sure that one had not developed. In the experiments of Robertson (1) as also in Rous' (6) study of induced auto-antibodies, incompatibility leading to destruction within the organism of transfused blood was regularly accompanied by an agglutination *in vitro* of the donor's corpuscles by the recipient's serum; while in those instances in which isoagglutinins were lacking no such destruction was evident. To make sure of obtaining wholly compatible donors, especially searching agglutination tests were now made. As a check upon the general findings one recipient was purposely given incompatible instead of compatible blood.

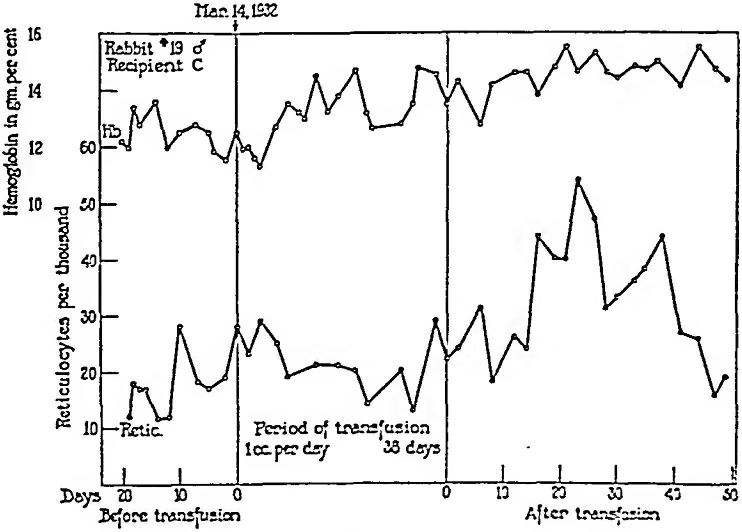

CHART 4

The donors were vigorous animals which it was supposed would easily bear the loss of 1 cc. of blood *per diem*, as indeed proved to be the case. The condition of their blood was studied with the same care as was that of the recipients. Frequent hematocrit determinations of the red cell bulk were made in both groups. The observations were kept up for weeks after the transfusions had been discontinued.

Five compatible pairs of animals and one incompatible pair were employed. For the selective agglutination tests, the serum of the recipients was mixed with the washed cells of the donors, 9 parts of serum to 1 of a 50 per cent suspension of the cells in salt solution. After 2 hours at room temperature, the mixtures were examined in the gross and microscopically. There was, in the case of the incompatible pair, well marked gross and microscopic agglutination of the donor's cells. As an accessory check upon the development of incompatibility during the transfusions, specimens of the blood of the recipients were examined for auto-agglutination (7). None transcending the normal was found save in the case of the animal receiving incompatible blood.

The initial weights of the rabbits were from 1800 to 2400 gm., and they were weighed each week, the figures showing a steady, gradual gain, as great in the donors as in the recipients, the final range being from 2300 to 3200 gm. All were kept in individual cages and on the same diet as the preceding series, save that cabbage was given three times a week.

The hemoglobin percentage, the number of reticulocytes, and the red cell bulk were ascertained at frequent intervals, usually every other day, for a period of at least 20 days preceding the first transfusion; and the observations were kept up during the 37 days of transfusion and for 6, in some cases 8, weeks thereafter. The blood specimens were taken in the morning, prior to feeding, the transfusions being done between 11:30 and 12 noon.

No anticoagulant was employed in this series to keep the injected blood fluid. The donor was placed in a covered box from which its head projected and the recipient in another by its side on the laboratory table. The shaved and oiled ear of the donor was heated until an active circulation had developed. A small cut was made in one of the marginal veins of the ear, and as the blood gushed forth it was steadily drawn up into a tuberculin syringe. When 1 cc. had been obtained in this manner, all further bleeding was prevented by an assistant and the injection into the ear vein of the recipient was quickly made. In this way no time was lost, the interval from the nicking of the donor's ear vein to the completion of the transfusion averaging not more than 60 seconds. Such care was taken for hemostasis that usually the donor lost no more than the desired 1 cc. of blood, at most not more than a drop. It has been found possible to extend the method to the transfusion of larger amounts; but when these exceed 4 cc. the risk of clotting becomes great. The first 2 or 3 cc. are much more rapidly obtainable than larger amounts, owing to contraction of the vessels of the bleeding ear. This caused trouble in some later experiments. For the determinations of red cell bulk the Van Allen hematocrit was used, with normal saline as the diluting fluid. Care was taken so to prepare this latter that changes in cell bulk due to osmotic imbalance were excluded. The findings during the transfusion and after periods showed that the red cell bulk varied directly with the hemoglobin quantity, as one might have expected under the conditions. Hence they are only occasionally charted.

In the analysis of the charts the experiment can be divided into three stages. There was the control period after the animals had been selected from stock, during which they lived under the same conditions as obtained subsequently when they functioned as donor and recipient. Then followed a period of 37 days, on each of which the recipient received from his paired donor 1 cc. of whole blood. And there was a post-transfusion period of observation ranging from 40 to 50 days (see Chart 3, Rabbit 25). The preliminary hemoglobin amount of the recipients ranged from 12 gm. per cent to about 15 gm., averaging 13 gm. That of the donors had the same range but was slightly less, averaging about 12.4 gm. when the transfusions were started. They were not begun until the fact was plain that the amount of blood pigment was practically constant from day to day.

Charts 3 and 4 are typical of the findings in the group of animals receiving 1 cc. of compatible blood per day, an amount approximating 1/100 of their own initial quantity. There occurred in every instance (see Chart 5 giving averages) a progressive increase in hemoglobin percentage, gradual, as one would expect under the circumstances, but continuous, the average increase being about 2 gm. during the 38 transfusion days, the consequence being that sometimes the ultimate circulating amount far exceeded that encountered in any normal rabbit by the author. For example the rise in Rabbit 25 (Chart 3) was from an average of 15 gm. to one of nearly 17 gm. The reticulocyte percentage remained essentially unchanged throughout the transfusion period, a fact shown for the group as a whole by Chart 5.

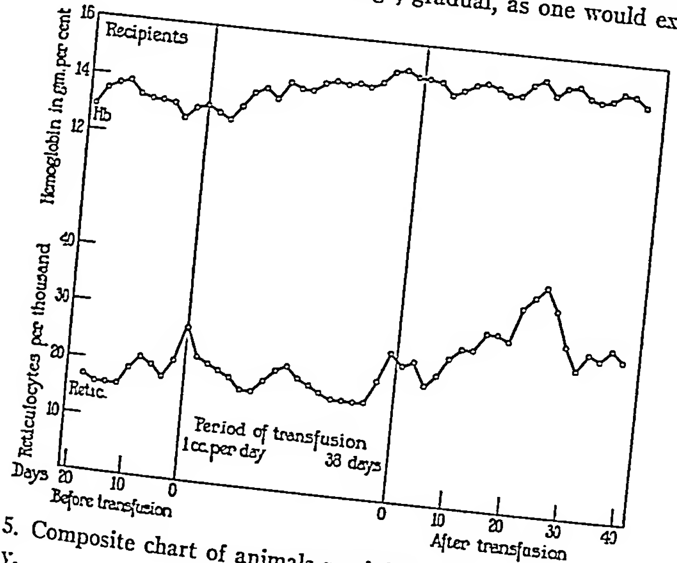

CHART 5. Composite chart of animals receiving small amounts of compatible blood daily.

Analysis of the Findings in the Recipients of Blood

The fact that the hemoglobin percentages rose markedly in the recipients does not necessarily mean that the pigment was present in superabundance. The rises might conceivably have been the result of a diminished blood volume. But there are observations on record, notably the extensive ones of Boycott and his coworkers, which prove that after transfusion the blood volume returns to normal or is only slightly increased. A lessening in volume seems never to have been observed.

A second possibility has been mentioned already; namely, that the increase in hemoglobin was within the bounds of the normal, representing merely a blood betterment consequent upon favorable conditions. The experiment just described was designed to exclude this possibility, through the selection as recipients of rabbits having the highest initial amounts of hemoglobin encountered in normal animals. It is, of course, impossible to state precisely the upper limit of the normal, but it is sufficiently demarcated for present purposes by the data of Pearce and Casey (8), who themselves worked with Rockefeller Institute stock. The figures on blood hemoglobin that they obtained by the examination of 174 animals procured from stock range from 28 to 90 per cent (Newcomer, 4.7 to 15.2 gm. with the instrument Pearce and Casey employed); but pathological instances were not ruled out, a fact sufficiently obvious from the figures. The distribution frequency of the curve representing the individual findings, as also the data of a subsequent paper, yield a maximum and minimum range for the generality of animals of from 52 to 74 per cent (8.8 to 12.5 gm. per cent). The first group of "normal" animals of the present work had initial hemoglobin values of 9 to 11 gm. per cent and those of the third group from 12 to 15 gm. with an average of 13 gm. It is evident that prior to transfusion the first group of animals utilized as recipients had hemoglobin values somewhat above the lower level of the normal while those of the third group, which had been selected for high hemoglobin values, were at or near the normal maximum. In these latter animals a further considerable increase occurred during the transfusion period, one which in some instances definitely transcended the individual maximum encountered by Pearce and Casey. Such an increase can justly be regarded as constituting superabundance.

The mounting curves expressive of the hemoglobin percentages in the individual charts yield no indication of a "ceiling," such as could be taken to represent the upper limit of the normal, beyond which obstacles to the increase in pigment might conceivably be encountered. In Rabbit 25, Chart 3, the curve followed the same slant in reaching 17 gm. per cent as in Nos. 1 and 2 in which it attained to only 13.8 gm. per cent and 14 gm. per cent respectively.

In what way did the increase in hemoglobin come about? There are several possibilities. One might suppose that the daily addition of blood from without was greater than that provided by the marrow and that this tissue ceased to work, a fact masked by the persistence in circulation of the reticulocytes already present or injected with the strange blood. But reticulocytes do not persist as such in the circulation. Boycott and Oakley (2) have reviewed the numerous papers which go to show that those of the rabbit mature into ordinary red cells in from 1 to 2 days after they leave the marrow. Those introduced with transfused blood disappear rapidly. Otherwise the count of such cells could not drop to zero as it frequently does for a brief period in rabbits receiving massive transfusions (1, 2). The daily introduction of 1 cc. of blood into the recipients of the present experiments would not suffice of itself to provide enough reticulocytes to maintain the count even if those introduced persisted as such throughout the transfusion period.

It is possible to calculate the total number of reticulocytes transfused in those instances in which their number was followed in the donors (Charts 3 and 4). For example in Recipient C, the reticuloycte count after 30 days when the hemoglobin had increased from 12.4 gm. per cent to 14.6 gm. per cent was still 3 per cent, about what it had been at the beginning. During this period the animal had received 30 cc. of strange blood from Donor C, carrying an average of 2.5 per cent of reticulocytes. This was introduced into a blood bulk of 113 cc. (since the rabbit has about 4.7 per cent of its weight in blood (9)). Assuming that all of the introduced reticulocytes persisted as such throughout the transfusion period, the gradual accumulation of them would account at most for only one-fourth the number in circulation at the end of the transfusion period. And the necessary assumption is not justified.

One is forced to conclude that the continued presence of reticulocytes in normal number throughout the transfusion period resulted from a persisting activity on the part of the erythropoietic tissue.

Granting that the marrow continued to work, to what was its work due—to lack of sensitiveness to the gradual increase in circulating blood pigment, as represented by the strange red cells, or to stimulation resulting from the intercurrent destruction of these cells? Care had been taken to provide compatible cells, yet many of them must have been destroyed in the natural course of events, together with the cells of the host; and they might even have been destroyed practically at once after introduction, a fact concealed through the activity of the erythropoietic tissue to form new ones.

The total increase in circulating hemoglobin was far from being as great as it should have been had none of the introduced blood been destroyed and the blood volume remained constant. The average final weight of the five rabbits of Chart 5 was 2580 gm. Assuming that the proportion of the blood volume to the weight remained stationary, and that it amounted to 47 cc. per kilo, the total increase in hemoglobin at its height represented an accretion of only about 18 cc. of blood, whereas 37 cc. had been injected. Boycott and Oakley found that large transfusions never raised the hemoglobin to the figure it should have reached on calculation, a finding which may have been due in some small part to an increase in the total blood volume, as may that now under discussion. The daily wastage from normal blood destruction, as shown by the bilirubin output, varies directly with the quantity of red cells in circulation (10).

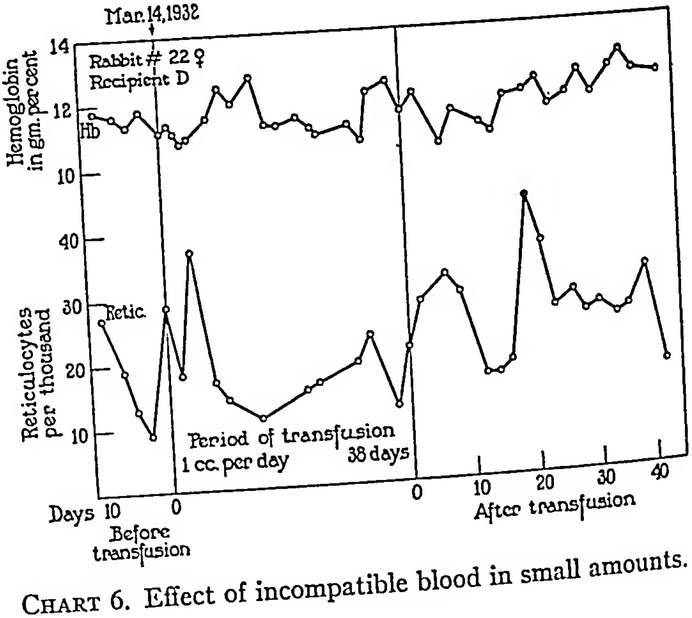

CHART 6. Effect of incompatible blood in small amounts.

Boycott and Oakley have reviewed the contradictory literature dealing with the question of whether the products of blood destruction act to stimulate the marrow. The fact has many times been proved that when the body needs blood the introduction of materials that can be used in its formation, laked blood for example, results in an increased activity on the part of the erythropoietic tissue. Indeed McMaster and Haessler (11) have shown that increases in the amount of this tissue to meet the emergency of anemia from hemorrhage are directly conditioned by the availability to it of the materials for blood formation. But increased reparative activity consequent upon the availability of such materials is not necessarily the same as direct marrow stimulation. Boycott and Oakley themselves found no such stimulation as result of the products of blood destruction save in a special instance, that of citrated, laked blood injected subcutaneously, laked blood as such failing of effect. Robertson did not obtain stimulation by transfusing incompatible blood to rabbits. As a check upon the possibility that in the present experiments the cells transfused were destroyed soon after introduction and utilized in the formation of new blood, agglutination tests of the third series of animals were made from time to time throughout the transfusion period to find whether the recipients had reacted against the donors' blood so that it had become incompatible. As already stated, it has been the experience of those working with rabbits that when such evidence of incompatibility fails to appear, transfused blood is well tolerated. In the present instances agglutination was not encountered. As a further check a rabbit was transfused with frankly incompatible blood to learn whether its destruction would lead to a mounting hemoglobin percentage. That in this instance the strange blood was promptly destroyed can be inferred from the fact that the hemoglobin underwent none of the increase seen in the animals receiving compatible blood (Chart 6); yet the reticulocytes remained at the pretransfusion level. Throughout the transfusion period there was a lack of significant intercurrent variations in the number of these cells, such as might have been expected had the marrow been subjected to restimulation.

From all this it seems plain that the marrow activity during the period while the hemoglobin was increasing, must have been due, not to stimulation by the products of blood destruction, but to persistence at a normal task.

The Effects of Repeated Small Blood Losses

The lack of sensitiveness of the marrow to induced increases in hemoglobin contrasts strikingly with its response to slight blood losses. It has been said that in the best controlled experiment, that of the third series of animals, observations were made on the donors at the same time as on the recipients. The loss sustained by these donors did not exceed by as much as a drop per day the amount of blood gained by the recipients. Yet in all six instances (Charts 7, 8, and 9) the percentage of reticulocytes markedly increased within a few days after the bleedings had been begun, and though the blood losses caused but a transient fall, when any, in the hemoglobin percentage, and were soon followed by recovery to a higher percentage than before, the reticulocyte count persisted above the previous level so long as blood was withdrawn. When no more was taken, the count soon fell, and reached the pretransfusion level. It is plain that not only did the erythropoietic tissue become abnormally active under the stimulus of the bleedings, but it never adjusted itself to the daily loss, being quite as sensitive thereto at the end of the period of hemorrhage as at the beginning.

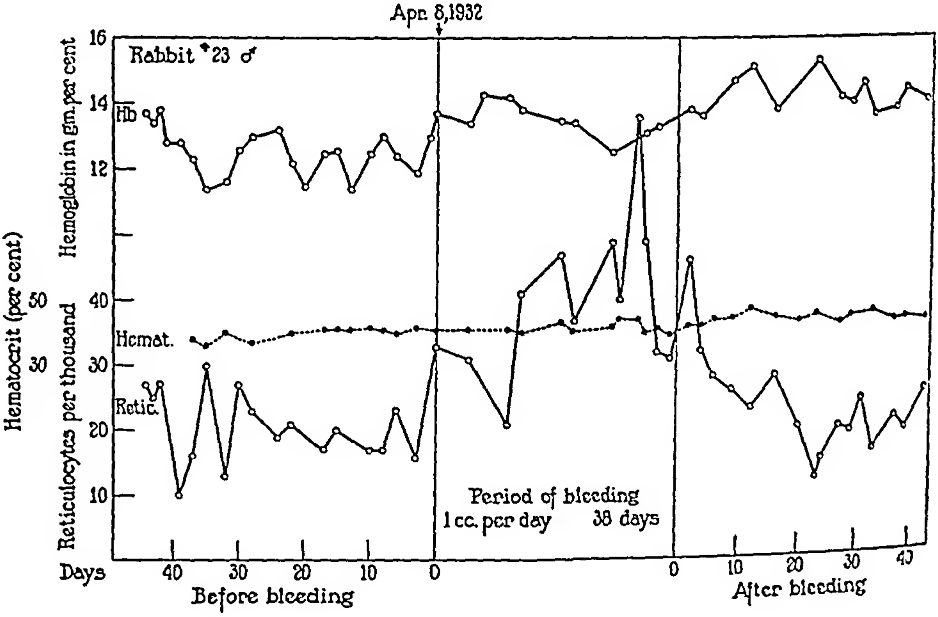

CHART 7

The hemoglobin percentage of the donor rabbits,—which throughout remained in excellent condition,—continued to rise progressively after the bleedings had been stopped, surpassing the previous normal, but not attaining the level reached in some of the recipient rabbits. This rise may conceivably have been due to overcompensation, such as is frequently observed after hemorrhage, though the effect of this does not ordinarily persist for so long a period.

The observation that daily small blood losses in healthy animals may not only be repaired, but may be attended by an increase in the amount of circulating hemoglobin, assumes special significance when the consequences are studied of removing the same total amount of blood on fewer occasions. The experiment was carried out on four rabbits selected and studied with the donors of the third experiment but deprived of 7 cc. of blood at a single bleeding once a week. In all four the reticulocyte percentage rose far more markedly than in the animals bled 1 cc. per day, yet the manufacture of blood was insufficient to compensate for the weekly loss and an anemia developed (Charts 10 and 11). The chart of the average findings (Chart 11) typifies the individual instances. There was a far greater increase in the number of reticulated cells than when the blood was withdrawn as 1 cc. per day. The blood removed was in one case injected into another, compatible rabbit. The hemoglobin of this animal was increased from 13.0 gm. to 14.2 gm. and a pronounced depression of marrow activity took place as evidenced by the reticulocyte change.

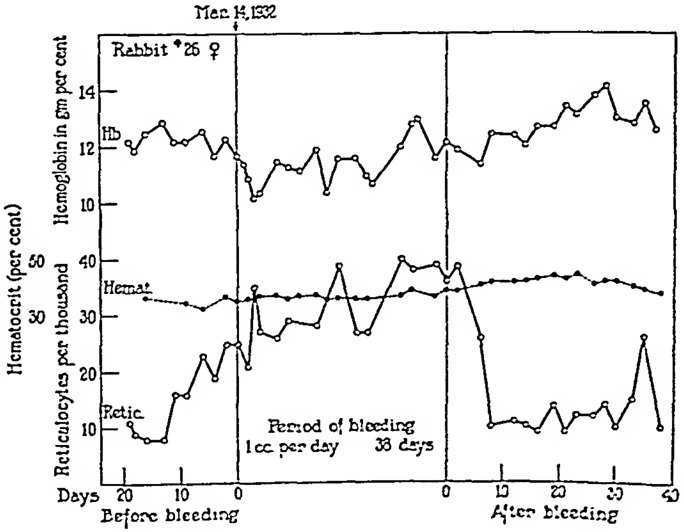

CHART 8

Habituation to Overmuch Hemoglobin

The fact that the erythropoietic tissue is insensitive to gradual additions to the amount of circulating blood pigment, while responding practically at once to withdrawals of the same magnitude, was evident in the present experiments not only during the period of transfusion, or of bleeding, but in the subsequent weeks, during which the blood studies were continued. For some days after the transfusions of 1 cc. of blood had been stopped the induced superabundance of hemoglobin persisted without change. Then the pigment percentage began to fall; and soon afterwards the erythropoietic tissue became unusually active, —as shown by a sharp rise in the reticulocytes,—and the high percentage was regained. This happened in the rabbits of all three series, but it was best studied, because best controlled, in the rabbits of the third group that received compatible blood (Charts 3, 4, and 5). The phenomenon occurred in all five of these animals. The high hemoglobin level was maintained throughout some weeks of observation, that is to say until expediency rendered it necessary to terminate the experiments; and the induced marrow activity, though not so great as after the hemoglobin first fell off, only gradually diminished to the normal rate as indicated by the percentage of reticulocytes. Evidently the organism had been so altered by the experimental procedures that a much higher hemoglobin percentage than that obtaining prior to the transfusions was now normal to it.

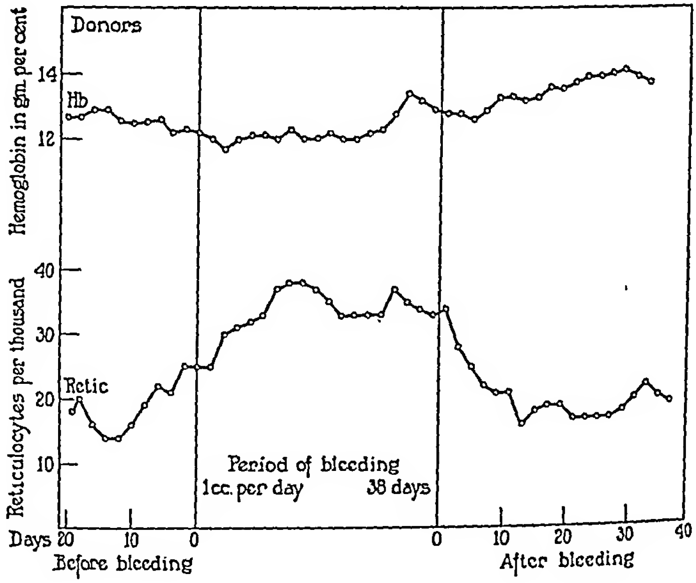

CHART 9. Composite chart of animals deprived of small amounts of blood daily.

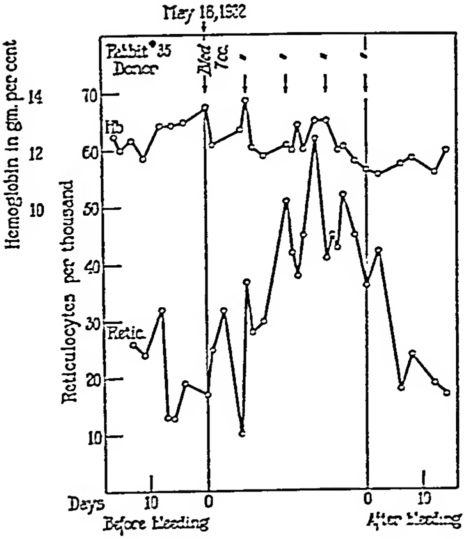

CHART 10

DISCUSSION

The initial object of the experiments was to determine the rate of blood production. It was supposed, on inference from indications here and there in the literature, that this could be done by supplying compatible blood to the normal organism in precisely the amount daily produced by the erythropoietic tissue, whereupon presumably this tissue would cease work. What occurred was wholly different. The day to day introduction into the circulation of small amounts of blood was without perceptible influence on bone marrow activity and the hemoglobin percentage rose gradually yet markedly. Its activity continued unabated throughout the transfusion period and must have contributed in no small part to the rise in hemoglobin. The grounds for these conclusions have been considered in the text.

If, in some of the recipient animals, it is possible to suppose that the increase in hemoglobin represented merely blood betterment over a previous low normal as result of favorable conditions (Charts 1 and 2), in the case of others (Charts 3 and 5) one is forced to look upon the state of affairs brought about by the additions of blood as constituting a superabundance for reasons that have been given. The findings as concerns marrow activity were similar in all of the recipients of small amounts of blood. They indicated that blood formation was practically unaffected by the gradually mounting hemoglobin (Charts 3, 4, and 5).

As already stated, Boycott and Oakley (2) transfused rabbits repeatedly with large amounts of blood to determine whether, as result of the provision of cells from without, the marrow would not atrophy or at least stop work. They found, as had Robertson before them, that the reticulocytes soon fell to zero; but although the transfusions were kept up this change did not endure but with every recurrent fall in hemoglobin from the abnormally high level,—and these were frequent,—the reticulocytes reappeared in greater or less number. Boycott and Oakley did not ensure compatible blood by preliminary tests; and the difficulties they mention of maintaining a superabundance of hemoglobin, difficulties evident in their charts, indicate that now and again the alien corpuscles must have been abruptly destroyed in quantity, a fact which they themselves recognized, doing control experiments on the possible effects of blood destruction to cause marrow stimulation. They could not bring the marrow completely to a standstill, though, as they remark, the abundance of transfused cells might be presumed to have saved the organ from the necessity of making any fresh ones.

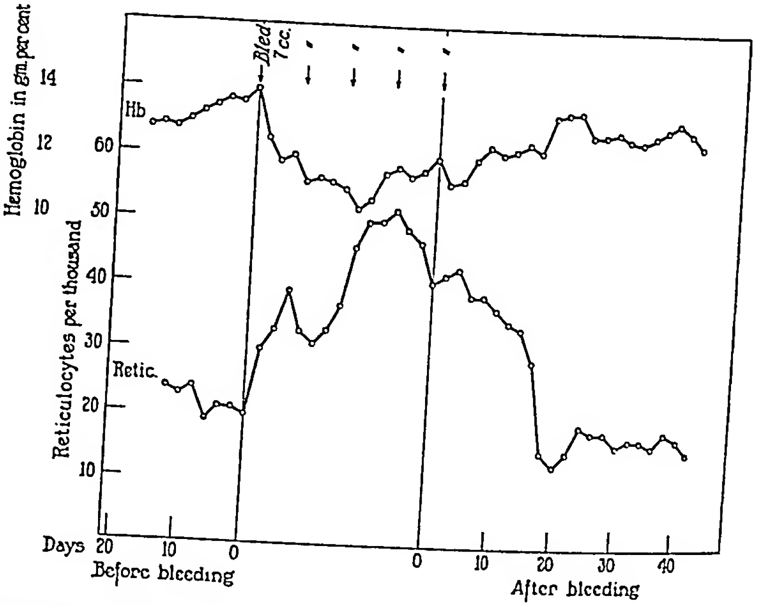

CHART 11. Composite chart of animals bled at weekly intervals.

Evidence was obtained in all of the recipient groups of the present work that habituation develops to an increased amount of circulating hemoglobin when this increase is brought about gradually. Soon after the daily transfusions were stopped, the hemoglobin percentage began to decline from its high level, but long before it had progressed far in the direction of the previous normal, the marrow became unusually active, as shown by a pronounced increase in the number of reticulated cells, and the high pigment level was restored and maintained (Charts 3, 4, and 5). The possibility that the increased erythropoietic activity resulted from the stimulation due to intercurrent blood destruction can be dismissed for reasons already given. Unfortunately the animals had to be sacrificed within a few weeks after the transfusions had been stopped, so the late consequences of these remain unknown.

Robertson showed that when a superabundance of hemoglobin had existed for some time the withdrawal of an amount of blood insufficient to bring the amount of pigment to the pretransfusion level resulted nevertheless in an increase in bone marrow activity. In doing this experiment he abruptly and considerably reduced the blood bulk. That considerable blood reductions stimulate the marrow of the normal animal, as repeated small losses to the same total amount do not, is shown by the large increase in the reticulocytes in our rabbits that were bled 7 cc. once a week as compared with the relatively slight increase in those bled 1 cc. *per diem*; and it might be inferred that Robertson's finding was the result merely of a drastic change in the blood bulk. This possibility was controlled in the transfused rabbits of the present work. Hemorrhage was not the cause of the post-transfusion fall in hemoglobin which resulted in marrow stimulation, nor, for that matter, was the reduction in the pigment percentage a great one.

Subsidiary evidence on the insensitiveness of the marrow to hemoglobin increases when these occur gradually can perhaps be found in the data on the donors for the third group of transfused rabbits. Although these lost 1 cc. of blood per day the hemoglobin percentages had returned to the initial level when the losses were stopped, and soon thereafter they rose above it. Thoughout the period of the bleedings the marrow had been unusually active as shown by the increased percentage of reticulocytes; but when they were discontinued, the number of these cells declined to the previous normal. Below this normal they did not fall despite the fact that the hemoglobin percentage continued to mount.

sponding to the repeated slight diminutions in blood bulk as such. The great response to bleeding 7 cc. once a week, as compared with 1 cc. per day, and the differing course of the hemoglobin curves, illustrate a point which deserves reiteration; namely, that the consequences of considerable and abrupt changes in blood volume and hemoglobin percentage provide no sufficient basis upon which to predict the outcome of repeated small ones to the same total amount.

The facts do not enable one to say whether the rabbits manifesting habituation to an increased amount of hemoglobin would have continued to keep this amount in circulation for a long period. Plainly the change in their circumstances had greatly altered their case. But whatever this case the hemoglobin percentage maintained by them would, in the absence of abnormal blood destruction, necessarily have been, in last analysis, the resultant of the forces making for depression and stimulation of the marrow respectively, just as in normal animals. According to Boycott and Oakley "The normal animal is evidently working about a nice level of delicate balance, which is presumably the reason for the constant presence of a few reticulated cells." A balance there certainly is, but hardly a nice one. For the present work has disclosed the fact that the erythropoietic tissue is insensitive to the effects of repeated, small blood accretions, though very sensitive to blood losses of the same magnitude. This is what one would expect if, throughout the course of age-long differentiation and selective survival, body needs have had the effect of determining body capabilities. The normal organism has always had to cope with accidental losses of hemoglobin, if it was to survive, but almost never with a superabundance of the pigment.

SUMMARY

The effects of very gradually increasing or diminishing the amount of circulating hemoglobin have been studied in rabbits. Contrary to expectation it was found that when the pigment was increased by the injection of a small quantity of compatible blood every day during some weeks the erythropoietic tissue did not lessen its activities. The hemoglobin percentage mounted gradually yet considerably when even as little as 1/100 of the amount of blood initially possessed by the animal was injected each day; and the figure it finally attained must in

some instances at least have been expressive of a superabundance. To this superabundance the animal itself evidently contributed through its persisting erythropoietic activity.

The results were very different when rabbits were bled daily to the same small amount that was injected into their fellows. The marrow became abnormally active, and this activity continued undiminished throughout the long period of the bleedings. The organism is evidently far more susceptible to blood losses than to blood gains, a fact which is scarcely surprising when one considers that throughout its differentiation as a going concern it has had to cope with exigencies of the first sort only.

Rabbits in which the hemoglobin is very gradually increased by the injection of strange blood become so accustomed to the abundance of pigment that even a slight falling off causes the erythropoietic tissue to become abnormally active to maintain the new *status quo*. Good reasons exist for referring the habituation thus manifested to readjustments in the functioning of the physiological mechanisms which mediate between oxygen demand and erythropoietic response. Too little recognition has been given to the rôle of these mechanisms in such relation. No evidence was obtained of any effective readjustment to protect the erythropoietic tissue from the stimulus of daily small blood losses.

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CHEMOIMMUNOLOGICAL STUDIES ON THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS

I. The Isolation and Properties of the Acetyl Polysaccharide of Pneumococcus Type I

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During the past several years it has become increasingly evident that Pneumococcus Type I possesses type-specific immunological properties not completely accounted for by those of the capsular polysaccharide in the form in which it was first chemically isolated and identified in this laboratory as the soluble specific substance (1–3). This fact was foreshadowed in the earlier studies of Perlzweig and his coworkers (4, 5), and has since been amply demonstrated by a number of European and American investigators, notably by Schiemann and his collaborators (6, 7) abroad, and by Enders (8, 9), Sabin (10), Wadsworth and Brown (11, 12), Ward (13) and Felton (14) in this country.

In view of this evidence, a further study has been made of the chemical and immunological properties of the soluble specific substance of Pneumococcus Type I, with the hope of acquiring a fuller knowledge of the nature of the relationship existing between the specifically reacting derivatives studied by other investigators and the type-specific polysaccharide previously described in this laboratory. The results of this study form the subject matter of the present report.

Evidence will be presented that the soluble specific substance has now been isolated in a chemical form more closely approximating that in which it exists as a natural constituent of the cell capsule. It will be shown that the type-specific carbohydrate present in the intact bacterial cells, and in filtrates of autolyzed broth cultures has been chemically identified as an acetyl polysaccharide. This naturally occurring acetyl polysaccharide differs chemically from the specific carbohydrate as originally isolated, principally in respect to the presence of acetyl groups which, as will be pointed out, endow the native substance with additional specific properties not possessed by the polysaccharide after removal of these labile groups by alkaline hydrolysis.

Owing to the marked instability of the acetyl groups and the ease with which they are removed by treatment with alkali, the soluble specific substance as originally isolated will be shown to represent the deacetylated polysaccharide which, although still retaining the dominant type specificity of the native substance, has through the loss of its acetyl groups suffered a corresponding loss of certain specific properties possessed only by the acetyl polysaccharide itself. Thus, specific differences between the properties of the cell fractions studied by other investigators and those of the soluble specific substance as originally defined, now appear to be due to the presence or absence of acetyl groups in the polysaccharide molecule. Indeed, so distinctive are the immunological reactions of the acetyl polysaccharide and those of its deacetylated derivative, that it is now possible to clarify many of the apparently conflicting views still current concerning the nature and properties of the specific carbohydrate of Pneumococcus Type I.

I. Isolation of the Acetyl Polysaccharide of Pneumococcus Type I

Methods for the isolation of the acetyl polysaccharide differ from those previously described for isolation of the soluble specific substance, principally in the avoidance of the use of an excess of alkali in the chemical procedures employed. The following methods have been used in the recovery and purification of the acetyl polysaccharide from the bacterial cells and concentrates of autolyzed cultures.

1. Bacterial Cells.—The bacterial cells from 18 hour broth cultures of Pneumococcus Type I, grown in 3 liter lots, were collected by centrifugation. The unheated cells from each lot were taken up in 50 cc. of sterile saline and stored in the ice box until the bacteria from 50 liters had been collected. The suspension of partially autolyzed cells was then treated with 15 cc. of N/1 acetic acid and heated in an Arnold sterilizer for 30 minutes. The precipitate of coagulated protein and bacterial debris was removed by centrifugation, and washed several times. The washings together with the original supernatant were concentrated to 150 cc. *in vacuo.* 150 cc. of alcohol were added to the concentrated liquid. The copious precipitate, containing the specific polysaccharide, was separated by centrifugation, and the alcoholic supernatant liquid was discarded. The carbohydrate was dissolved in 75 cc. of water, 1 cc. of N/1 acetic acid was added and the small amount of insoluble precipitate which formed was separated and discarded. The polysaccharide was precipitated from the solution by the addition of an equal volume of alcohol, the precipitate was recovered and again dissolved in 50 cc. of water. The solution of polysaccharide at this point was clear and colorless. It gave none of the usual protein tests. The solution was acidified by the addition of 2 cc. of 5 N HCl and dialyzed until no chlorine ion was detectable in the dialysate. At this point, a small amount of degraded (deacetylated) polysaccharide which had separated from the solution was removed by centrifugation. The viscous, clear and colorless solution of the specific acetyl polysaccharide was precipitated by pouring it into 10 volumes of acidulated acetone. The polysaccharide was separated by filtration on a porous, sintered glass funnel, and was washed with absolute alcohol and ether. 0.5 gm. of dry substance was recovered.

2. *Autolyzed Broth Cultures*.—50 liters of 0.5 per cent dextrose broth were seeded with an actively growing culture of Pneumococcus Type I, and incubated at 37°C. for 5 days during which time the bacterial cells had undergone marked autolysis. The reaction of the culture fluid after growth and autolysis had occurred was distinctly acid. The 50 liters of autolyzed broth culture were autoclaved and concentrated to one-tenth of the original volume in a steam kettle. The concentrated material was cooled to 0° and neutralized, while stirring, with solid sodium bicarbonate, 6 liters of alcohol were added to the concentrate and after standing for 18 hours at room temperature, the clear, dark supernatant liquid was syphoned off. After removal of the excess fluid by centrifugation, the precipitate was dissolved as completely as possible in 800 cc. of water. The deep brown and turbid aqueous solution containing the pneumococcus polysaccharide was clarified by centrifugation at high speed. The precipitated bacterial debris and coagulated protein was next washed with slightly acidulated water, until the washings gave only a faint Molisch test. Four to five washings were found necessary. To the combined solution of polysaccharide and wash waters, now at a volume of about 1 to 1.2 liters, were added 5 cc. of N/1 acetic acid and 50 gm. of sodium acetate. A slight precipitate separated which was removed by centrifugation and discarded. 1.2 volumes of alcohol were now added to the supernatant and the precipitate was recovered by centrifugation. The precipitate was dissolved in about 250 cc. of water and 25 gm. of solid trichloracetic acid were dissolved directly in the turbid solution. After standing 2 hours at 0°, a heavy deposit of coagulated protein precipitated from solution and was separated by centrifugation. The clear, pale yellow and strongly acid supernatant liquid, containing most of the polysaccharide, was placed in the ice box. The precipitate of coagulated protein, still containing a considerable amount of adsorbed specific polysaccharide, was suspended in 100 cc. of water, cooled to 0° and brought as completely as possible into solution by the *cautious* addition of ice-cold 2 N NaOH. The solution was not allowed to become definitely alkaline at any time. A solu-

tion of 50 per cent trichloracetic acid was then carefully added until the point of maximum precipitation was reached. After standing 10 minutes, the coagulated protein was centrifuged and the supernatant liquid was added to that in the ice box. Solution and reprecipitation of the coagulated protein were repeated until the supernatant fluid gave only a faint or a negative Molisch test. A total of two or three reprecipitations sufficed. To the combined supernatant liquids containing the specific polysaccharide in a volume of about 500 cc., 20 gm. of sodium acetate were added and the solution was cooled to 0°. On the addition of 1.2 volumes of alcohol to the chilled solution, the polysaccharide precipitated as a white flocculent mass. The substance was collected in the usual way and dissolved in 100 cc. of water. After adjusting the reaction to approximately pH 4 and allowing the solution to stand for several hours, a small amount of insoluble residue was separated by centrifugation. 5 gm. of sodium acetate were added and the polysaccharide was precipitated by adding an equal volume of cold alcohol. The precipitate was separated, and dissolved in 75 cc. of water, yielding a clear and almost colorless solution which gave none of the usual protein tests. The material was made definitely acid to Congo red with HCl, and was dialyzed until no chlorine ions were detectable in the dialysate. A small quantity of deacetylated specific polysaccharide which had precipitated from the solution was separated by centrifugation. The viscous solution of acetyl polysaccharide was poured into 10 volumes of acidulated acetone. The precipitated carbohydrate was separated by filtration and washed with alcohol and ether. 2.1 gm. were recovered. The product thus obtained was nearly white in color. A 1 per cent solution gave no test for protein.

3. *Analytical Methods.*—The acetyl polysaccharide was analyzed for total nitrogen by a modification of the Pregl (16) method. Amino nitrogen was determined by the method of Van Slyke (17). Acetyl determinations were made on samples of 15 mg. by Pregl's method (18). The specific optical rotation was observed in a 2 dm. tube on aqueous solutions of known concentration. The acid equivalent of the specific acetyl polysaccharide was determined by titrating an aqueous solution of known concentration at 0° to pH 7 with N/50 NaOH. The acid equivalent of the deacetylated carbohydrate was determined by dissolving a weighed sample in a known quantity of N/10 HCl, then neutralizing the measured HCl with an equivalent quantity of N/10 NaOH, and finally titrating the finely suspended isoelectric polysaccharide to pH 7 with N/50 NaOH. Reducing sugars were determined by the Hagedorn-Jensen method (19) after hydrolyzing solutions of known concentration with 1.5 N HCl in sealed tubes at 100°.

II. Chemical Properties of the Acetyl Polysaccharide of Pneumococcus Type I

By the methods described above, in which treatment with alkali was purposely avoided, the soluble specific substance of Pneumococcus Type I has been isolated in the form of an ash-free acetyl polysaccharide possessing marked acidic properties. The specific carbohydrate in this form is very soluble in water, and gives solutions of high viscosity. Aqueous solutions show a specific optical rotation of about +270°. The naturally acetylated polysaccharide contains 4.85 per cent of nitrogen, approximately one-half (45 per cent) of which is liberated in the amino form when the substance is treated with nitrous acid in the cold. It does not reduce Fehling's solution until after hydrolysis with dilute mineral acids. At the same time that reducing sugars appear in the solution, the serological specificity of the acetyl polysaccharide is destroyed. The behavior in this respect is identical with that of the deacetylated polysaccharide.

On the addition of hydrochloric acid-napthoresorcinol, both forms of the specific carbohydrate give a marked color reaction indicating the presence of uronic acids. In addition to being soluble in water, the acetyl polysaccharide is soluble in 80 per cent acetic acid. Aqueous solutions of 0.5 per cent concentration are precipitated by the following reagents: phosphotungstic acid, silver nitrate, neutral and basic lead acetate; and are incompletely precipitated by barium hydroxide. Unlike the deacetylated product, the acetyl polysaccharide is precipitated by tannic acid but not by uranyl nitrate. It gives no color reaction with iodine-potassium iodide solution. Weak solutions of potassium permanganate are not immediately decolorized by the acetyl polysaccharide. The biuret, ninhydrin, sulfosalicylic and picric acid tests are negative. No traces of phosphorus or sulfur were detectable in the most highly purified preparations of the specific acetyl polysaccharide.

The following experiments were carried out, in order to identify the organic acid liberated from the naturally acetylated polysaccharide by alkaline hydrolysis, and to determine whether the chemical properties of the carbohydrate after deacetylation are identical with those of the specific polysaccharide formerly obtained by methods involving the use of alkali in the process of isolation.

1. Conversion of the Acetyl Polysaccharide to Its Deacetylated Derivative by Alkaline Hydrolysis

0.0693 gm. of acetyl polysaccharide (Preparation 2, Table I) was dissolved in 7 cc. of water and neutralized to phenolphthalein with 1.42 cc. of N/10 NaOH. 7 cc. more of N/10 alkali were added and the mixture was heated in a boiling water bath for 35 minutes. The reaction mixture was then neutralized and finally 1.5 cc. of N/10 HCl were added in excess. A precipitate appeared in the solution. The flask containing the mixture was placed in the ice chamber for 24 hours, after which time the precipitate was separated by centrifugation and washed several times with small quantities of ice water. 0.052 gm. of material was recovered. The substance was dried at 100° in high vacuum.

TABLE I

Analyses of the Acetyl Polysaccharide of Pneumococcus Type I

Preparation No.	Source	Acid equivalent	Specific rotation	Ash	C	H	Total nitrogen	Amino nitrogen	Acetyl	Phosphorus	Reducing sugars after hydrolysis	Highest dilution of polysaccharide reacting with antipneumococcus serum
				per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
1	Bacterial cells	–	+270°	0.0	–	–	4.89	2.30	5.9	0.0	–	1:5,000,000[*]
2	" "	–	+265°	0.0	–	–	4.86	2.21	6.9	–	–	1:5,000,000[*]
3	Autolyzed broth cultures	576	+277°	0.0	42.55	6.58	4.85	2.22	6.0	0.0	32.0	1:5,000,000[*]
2 A, deacetylated	†	535	+297°	0.0	‡	‡	5.05	2.50	0.0	–	27.6	1:5,000,000[§]

[*] Type I antipneumococcus serum previously absorbed with Preparation 2 A (deacetylated).

[†] This sample of deacetylated polysaccharide was obtained by alkaline hydrolysis of Preparation 2. This material is identical with the carbohydrate formerly known as the soluble specific substance of Type I Pneumococcus.

[‡] An analysis of carbon and hydrogen was made on a sample of deacetylated carbohydrate which had been reprecipitated five times at its isoelectric point. The material contained no ash, and had a carbon content of 40.33 per cent and a hydrogen content of 6.23 per cent.

[§] Unabsorbed Type I antipneumococcus serum.

The analytical data presented in Table I show that the deacetylated product (Preparation 2 A) obtained by alkaline hydrolysis of the acetyl polysaccharide (Preparation 2) contains no acetyl groups[1] and is in all respects chemically identical with the polysaccharide which has hitherto been known as the soluble specific substance.

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[1] Heidelberger and Kendall have previously found that the Type I polysaccharide (deacetylated) contains no acetyl groups. (See *J. Exp. Med.*, 1931, 53, Table III on page 636.)

2. *Identification of Acetic Acid in the Acetyl Polysaccharide of Pneumococcus Type I*

0.80 gm. of Preparation 3 (Table I) was dissolved in 25 cc. of water and neutralized. 25 cc. of N/1 NaOH were added, and the solution was heated in a boiling water bath for 40 minutes. The mixture was cooled and 6 cc. of 5 N H_2SO_4 were added. The solution was placed in a Claissen flask and distilled *in vacuo*. The delivery tube of the Claissen flask was so bent that it extended to the bottom of a receiving flask. The latter contained a suspension of freshly prepared and carefully washed silver carbonate, and the entire flask was packed in ice. The contents of the Claissen flask was distilled nearly to dryness, then 50 cc. of water were added and the distillation repeated. This was done three times in all. The receiving flask was then disconnected, and the excess silver carbonate was removed by filtration. The filtrate from the silver carbonate, which contained the silver salt of a volatile organic acid, was concentrated to 20 cc. *in vacuo*. The solution was warmed slightly and filtered. The filtrate was cooled to 0°, and 50 cc. of neutral, freshly distilled ethyl alcohol were slowly added. A snow-white product crystallized promptly from the solution. In crystalline form this material was identical with an authentic sample of silver acetate. The unknown silver salt was filtered, washed with a little dilute alcohol and dried *in vacuo* at 56° to constant weight; 0.080 gm. was recovered. The product was analyzed for silver by the usual method. The silver was weighed as silver iodide.

Analysis: 0.0483 gm. substance gave 0.0677 gm. AgI.

CH_3COOAg Calculated: Ag 64.7 per cent.
Found: Ag 64.4 per cent.

From the above analysis it is seen that the volatile organic acid liberated from the polysaccharide by alkaline hydrolysis is acetic acid, bringing chemical proof that the specific carbohydrate exists as an acetyl polysaccharide. The exact mode of linkage of the acetic acid is not as yet definitely known. Judging from the ease with which the acetyl groups are removed by dilute alkali, even at room temperature, it seems certain that the nitrogenous groups of the polysaccharide are not acetylated. It appears more likely that in the native polysaccharide the acetic acid is bound directly to the hydroxyl groups attached to a carbon atom.

The possibility remains, of course, that in addition to the acetyl groups, the specific carbohydrate, in the state in which it exists as a natural constituent of the capsular substance, may also have other labile groups attached to it. However, there is certain evidence which lends support to the view that the only chemical difference between the naturally acetylated and the artificially deacetylated polysaccharide lies in the presence of acetyl groups in the former substance. For it has been found that the acid equivalent of the acetyl polysaccharide is 576, while that of the deacetylated substance is 535, when each is titrated to pH 7 with N/50 NaOH. The difference in the observed values of the acid equivalent is 41, which is in close agreement with the theoretical value of 43, representing the difference required if the native polysaccharide contains one acetyl group per 576 of formular weight. The specific polysaccharide may therefore be regarded, tentatively at least, as an acetyl ester.

The chemical evidence thus far available indicates that the soluble specific substance of Pneumococcus Type I exists in its native state as an acetyl polysaccharide, and that the specific substance recovered by the earlier methods must now be regarded as the deacetylated derivative of the native carbohydrate from which the labile acetyl groups have been removed by treatment with alkali during the chemical manipulations incident to its isolation.

In the following experiments the immunological properties of the two forms of the specific polysaccharide and their serological relationships to each other will be considered in terms of these chemical findings.[2]

III. Immunological Properties of the Acetyl and the Deacetylated Polysaccharide of Pneumococcus Type I

The preceding experiments have revealed the chemical relationship existing between the naturally acetylated and artificially deacetylated form of the specific capsular polysaccharide of Pneumococcus Type I. In the following experiments these chemical differences are shown to be reflected in the immunological reactions of the two forms of the specific carbohydrate. The acetyl polysaccharide is found not only to fulfil all the immunological criteria of type specificity, but also to possess certain additional specific properties which the deacetylated polysaccharide lacks through the loss of the extremely labile acetyl groups.

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[2] The authors wish to express their appreciation to Mr. Frank H. Babers for his kindly cooperation in much of the analytical work and to Dr. Michael Heidelberger and Dr. P. A. Levene for their interest and helpful suggestions.

1. *Precipitation of the Acetyl and Deacetylated Polysaccharide in Absorbed and Unabsorbed Type I Antipneumococcus Serum*

Immune horse serum[3] was absorbed by the fractional addition of a 1:2000 solution of the acetyl polysaccharide, until the supernatant serum after removal of the successive precipitates no longer reacted on the further addition of the specific substance. An equal portion of the same lot of serum was similarly absorbed with the deacetylated polysaccharide.

10 cc. of antipneumococcus horse serum (Type I) were diluted with 8 cc. of salt solution. To the diluted serum, 1 cc. of 1:2000 solution of the specific polysaccharide was added. The mixture was incubated for 2 hours at 37° and then placed in the ice box for the same length of time. The precipitate was thrown down by centrifugation in the cold and the clear supernatant serum was pipetted off. This procedure was repeated three times in all. Finally, 0.5 cc. of the solution of polysaccharide was added to the serum and the mixture was incubated for 2 hours at 37° and then allowed to stand in the ice box 24 to 48 hours. After removal of the final traces of precipitate by centrifugation in the cold, the clear supernatant serum was pipetted off and made up to a volume of 25 cc. with salt solution, so that each 0.5 cc. of absorbed serum used in the tests equalled 0.2 cc. of original serum.

The original serum and the two separately absorbed portions of the same serum were tested for the presence or absence of precipitins for both forms of the capsular polysaccharide. The results of the precipitin tests are given in Table II.

From the data recorded in Table II it is seen that both the acetyl and the deacetylated polysaccharide were precipitated by the original, unabsorbed serum in the highest dilution tested, representing a final concentration of 1 part in 3 million. The serum absorbed with the deacetylated polysaccharide, after removal of all precipitins for this form of the specific carbohydrate, still reacted with the acetyl polysaccharide in equally high dilution. On the other hand, after absorption with the acetyl polysaccharide, the serum was completely exhausted of all precipitins for both forms of the carbohydrate, as shown by the lack of reaction when tested with each substance in dilutions ranging from 1:20,000 to 1:3,000,000. It is a significant fact that the

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[3] The antipneumococcus horse serum used in these experiments was provided through the courtesy of Dr. Augustus Wadsworth, Director of the Division of Laboratories and Research, New York State Department of Health, Albany.

deacetylated polysaccharide selectively takes out from the serum only the precipitins for itself, whereas the acetyl polysaccharide completely removes all the precipitating antibodies for both forms of the specific substance.

Enders (8) (1930) demonstrated that there exists in the autolytic products of Pneumococcus Type I a substance which is specifically precipitable in immune serum devoid of all antibodies for the soluble specific substance (deacetylated). He further showed that this material after being heated in a weakly alkaline solution lost its capacity to react in the same serum. The substance therefore appeared to be so sharply differentiated by its immunological reactions and its instability to alkali, that Enders considered it to be quite distinct, and provisionally called it "the A substance" to distinguish it from the specific carbohydrate. Wadsworth and Brown (11, 12) (1931, 1933) isolated from the bacterial cells a substance which, like the A substance of Enders, precipitated with Type I antipneumococcus serum that had previously been absorbed with the soluble specific substance (deacetylated). They also found that the substance designated by them "the cellular carbohydrate," when boiled for 2 minutes in N/100 NaOH no longer reacted in the absorbed serum, indicating, as the authors suggest, that boiling in alkaline solution had so altered their original material that its activity under these conditions approximated that of the soluble specific substance (deacetylated).

TABLE II

Precipitation of the Acetyl and Deacetylated Polysaccharide of Pneumococcus Type I in Homologous Antiserum before and after Absorption of the Anticarbohydrate Precipitins

Antipneumococcus Serum Type I	Acetyl polysaccharide					Deacetylated polysaccharide				
	1:20,000	1:100,000	1:500,000	1:1,000,000	1:3,000,000	1:20,000	1:100,000	1:500,000	1:1,000,000	1:3,000,000
Unabsorbed	++++	++++	+++±	+++	++	++++	++++	++++	+++	++
Absorbed with deacetylated polysaccharide	++++	++++	++±	++	+	–	–	–	–	–
Absorbed with acetyl polysaccharide	–	–	–	–	–	–	–	–	–	–

++++ = complete precipitation, compact sediment with clear supernatant.

– = no precipitate formed, fluid clear.

The final readings were made after incubating the reacting mixtures 2 hours at 37° and overnight in the ice box.

The specific precipitation of the acetyl polysaccharide in serum previously absorbed with the deacetylated carbohydrate, and the readiness with which the former substance is converted into the latter by alkali and heat, are similar to the relationships observed by Enders (8), and by Wadsworth and Brown (12), between the substances isolated by them and the soluble specific substance which they prepared according to methods previously described in this laboratory. Since the specific substance thus prepared is now known to be the deacetylated polysaccharide, it seems not improbable that the differences they observed, like those noted in Table II, represent the reactions not of two different carbohydrates but of a single substance in two chemically different forms; namely, the naturally acetylated and the artificially deacetylated polysaccharide.

2. *Agglutination of Type I Pneumococci in Homologous Antiserum before and after Absorption with the Acetyl and Deacetylated Polysaccharide*

Type I antipneumococcus serum was separately absorbed with the acetyl and the deacetylated polysaccharide as previously described. The results of the agglutination tests of Type I pneumococci in homologous antiserum before and after specific absorption are given in Table III.

The experimental data presented in Table III show that absorption of Type I antiserum with the acetyl polysaccharide completely removed all the type-specific agglutinins, as evidenced by the fact that, after absorption, the serum no longer agglutinated the homologous organisms. On the other hand, the serum similarly absorbed with the deacetylated polysaccharide still agglutinated the bacteria although the titer of agglutinins was considerably reduced. The fact that after absorption with the deacetylated polysaccharide the precipitin titer appeared undiminished for the acetyl polysaccharide, while the titer of agglutinins for the bacterial cells was reduced, may be attributed not to essential differences in the antibodies involved in the two forms of immune reactions, but to differences in the technical procedures of diluting the antiserum in the agglutination test, and of maintaining an excess of serum throughout the range of the precipitin titration. Of special significance in the present study is the fact that the acetyl polysaccharide by itself completely exhausted the serum of all demonstrable type-specific precipitins and agglutinins.

Sabin (10) and Enders (9) previously demonstrated that Type I antiserum after absorption with the specific carbohydrate (deacetylated) still agglutinated pneumococci of the homologous type. Enders further showed that when the bacterial cells were heated in a weakly alkaline medium, they lost the capacity to react in the absorbed serum although in this altered state they were still specifically agglutinated by the original, unabsorbed serum. The fact that the bacteria were agglutinated by immune serum containing no antibodies reactive with the specific carbohydrate (deacetylated), and the further observation that after boiling for 10 minutes at pH 8.8 the cells no longer reacted in this same serum, led Enders to the conclusion that there exists a type-specific substance distinct from the specific carbohydrate in Pneumococcus Type I.

TABLE III

Agglutination of Type I Pneumococci in Homologous Antiserum before and after Absorption with the Acetyl and the Deacetylated Polysaccharide

Antipneumococcus Serum Type I	Agglutination of Pneumococcus Type I in serum dilutions						
	1:10	1:20	1:30	1:40	1:60	1:80	1:100
Unabsorbed	++++	++++	++++	++++	+++	++	+
Absorbed with deacetylated polysaccharide	++++	++	+±	±	−	−	−
Absorbed with acetyl polysaccharide	−	−	−	−	−	−	−

++++ = complete agglutination, compact sediment with clear supernatant.
− = no agglutination.

As pointed out earlier in this paper, the acetyl polysaccharide is readily converted into the deacetylated substance by treatment with alkali. This fact, together with the observations just cited on the serological reactions of the two forms of the specific polysaccharide, not only substantiates the findings of the former investigators but also furnishes evidence of the immunological significance of this hitherto unrecognized relationship. Thus, on the basis of the present evidence, it appears that the acetyl polysaccharide represents the soluble specific substance in a form that fulfils all the serological requirements of type specificity without the necessity of predicating a second substance distinct from the specific carbohydrate itself. These observations are further confirmed by the results of the following protection experiments.

3. *Protective Action of Type I Antipneumococcus Serum before and after Absorption with the Acetyl and Deacetylated Polysaccharide*

Protection tests in mice were carried out according to the method described by Felton (15).

Dilutions of the unabsorbed and absorbed serum, calculated on the basis of original serum volume, were made, ranging from 1:10 to 1:500. 0.5 cc. of each dilution of serum together with 0.5 cc. of 1:200 dilution of a 12 hour plain broth culture of Pneumococcus Type I was injected intraperitoneally into white mice weighing from 18 to 21 gm. The virulence of the organisms was such that 10^{-5} cc. of culture caused the death of normal control mice in 48 hours. All mice alive and well 7 days after inoculation were considered effectively protected and were recorded as survivals.

The results of experiments to determine the protective action of Type I antipneumococcus serum before and after absorption with the acetyl and deacetylated polysaccharide are given in Table IV.

The outcome of the protection tests (Table IV) shows that after absorption with the deacetylated polysaccharide, the serum still possessed protective action, although the titer of protective antibodies was considerably reduced. In an earlier study of the neutralizing effect of the soluble specific substance, Sabin (10) showed that this substance, which in the light of the present results was presumably in the form of the deacetylated polysaccharide, only partially neutralized the protective power of Type I antipneumococcus serum. He attributed the residual protective action of the absorbed serum to the presence of type-specific antibodies not neutralized by the homologous soluble specific substance and distinct from the anticarbohydrate precipitins. The comparative data presented in Table IV show that the soluble specific substance in the form in which it naturally occurs as the acetyl polysaccharide completely removed the protective antibodies in Type I antiserum. This neutralizing effect is shown by the fact that after removal of all the anticarbohydrate precipitins by absorption with the acetyl polysaccharide, the immune serum was devoid of protective action when titrated by the method employed in the present experiments.

These results again emphasize the relationship existing between the natural acetyl polysaccharide and its deacetylated derivative. It now becomes apparent why the specific carbohydrate in the form in which it was first isolated was later found to be deficient in certain specific properties, notably in its failure to absorb completely the type-specific antibodies from immune serum. By the methods employed in the original isolation, the specific carbohydrate is now known to have been artificially deacetylated, and its immunological deficiencies have been found to be associated with the loss of the highly reactive but ex-

TABLE IV

Protective Action in Mice of Type I Antipneumococcus Serum before and after Absorption with the Acetyl and the Deacetylated Polysaccharide of Pneumococcus Type I

Dilution of serum	Antipneumococcus Serum Type I					
	Unabsorbed		Absorbed with			
			Deacetylated polysaccharide		Acetylpolysaccharide	
1:500	S	S	D 21	D 28	D 22	D 25
1:250	S	S	D 26	D 51	D 20	D 23
1:100	S	S	S	S	D 17	D 18
1:50	S	S	S	S	D 17	D 21
1:10	S	S	S	S	D 19	D 23

All mice were injected intraperitoneally with 0.5 cc. of diluted serum together with 0.5 cc. of 1:200 dilution of broth culture of Pneumococcus Type I.

Controls

Pneumococcus Type I	Mice receiving no serum
cc.	
10^{-6}	D 45
10^{-7}	D 45
10^{-8}	D 48

S = survived. D = died. Numerals indicate the number of hours elapsing before death of the animal.

tremely labile acetyl groups. The significance of this fact is made evident by the results of the preceding experiments, in which it has been shown that the acetyl polysaccharide with these chemical groups intact specifically bound and completely removed from the serum all the type-specific antibodies.

4. *Antigenic Action of the Acetyl Polysaccharide in Mice*

In order to determine whether the acetyl polysaccharide is capable of inducing active immunity against infection with pneumococci of the homologous type, the antigenicity of this form of the specific carbohydrate was tested in mice and its action compared with that of the deacetylated polysaccharide

Six mice were given three intraperitoneal injections, at 3 day intervals, of 0.5 cc. of 1:2 million dilution of Type I acetyl polysaccharide; another group of six mice was similarly treated with identical amounts of the deacetylated carbohydrate prepared by heating the original material in N/20 alkali for 30 minutes at 100°. 6 days after the last immunizing injection both groups of mice were infected by the intraperitoneal injection of a virulent culture of Pneumococcus Type I in amounts ranging from 10^{-5} to 10^{-7} cc., the maximum number of infecting organisms being 1000 times greater than that which proved fatal in the normal control mice.

The results of the experiments on the active immunization of mice with both forms of the specific carbohydrate are given in Table V.

As shown in Table V, the mice which had received in divided doses an amount of acetyl polysaccharide totaling only 0.00075 mg. of specific substance survived the injection of an amount of virulent culture of Pneumococcus Type I greatly in excess of that causing fatal infection in the untreated control animals. Repetitions of this test in mice have shown that the active immunity induced by the acetyl polysaccharide is strictly type-specific, affording no protection against infection with pneumococci of the heterologous Types II and III. It is equally clear from the results of this and other similar experiments that the deacetylated polysaccharide is wholly devoid of antigenic action. This total lack of immunizing effect is all the more striking in this particular instance, since the deacetylated substance was derived from the originally active acetyl polysaccharide by merely heating the latter in alkaline solution—a procedure previously shown to deprive the native carbohydrate of its acetyl groups.

During the past ten years a number of investigators using various methods have recovered from Pneumococcus, substances which have been shown to possess the property of inducing active immunity in mice against infection with organisms of the homologous type. In many instances, the antigenic and serological behavior of these substances was so distinct that the authors designated them by special terms in order to distinguish them from the soluble specific substance (deacetylated). Thus, there are now current in the literature descriptive terms such as the following: "the water-soluble fraction" of Perlzweig and his coworkers; "the A substance" of Enders; "the cellular carbohydrate fraction" of Wadsworth and Brown, and "the non-polysaccharide and probably non-protein derivative" of Felton. With the possible exception of the A substance, which Enders did not test for antigenicity in mice, these various cell derivatives have been found to produce type-specific immunity in this particular species of animal.

TABLE V

Active Immunity Induced in Mice by the Acetyl and Deacetylated Polysaccharide of Pneumococcus Type I

Amount of culture Pneumococcus Type I	Normal mice controls (untreated)	Mice receiving 3 injections of 0.5 cc. of 1:2 million solution of			
		Acetyl polysaccharide Type I		Deacetylated polysaccharide Type I	
cc.					
10^{-5}	—	S	S	D 68	D 68
10^{-6}	D 44	S	S	D 34	D 44
10^{-7}	D 52	S	S	D 58	D 76
10^{-8}	D 93	—	—	—	—

The treated mice were infected 6 days after the last immunizing injection.

— = not done.

S = survived. D = died. Numerals indicate the number of hours elapsing before death of the animal.

Since it is obviously impossible within the scope of this paper to review the individual contributions in detail, brief reference will be made only to those studies concerned with the antigenicity of specific

Perlzweig and Steffen (4) (1923) extracted from the bacterial cells a water-soluble fraction which induced specific immunity in mice. This observation was later confirmed by Ferry and Fisher (20) (1924, 1925) who obtained from washings of the organisms an aqueous extract which had similar antigenic properties in mice. The water-soluble antigen of Perlzweig and Steffen proved resistant to the prolonged action of autolysis and tryptic digestion. They further pointed out the suggestive fact that boiling the antigenic material for 5 minutes in alkaline solution (pH 9) destroyed its immunizing action in mice, while similar exposure to heat in a slightly acid medium (pH 6) did not impair its antigenicity. Perlzweig and Keefer (5) (1925) recovered from the filtrate of broth cultures a substance which, like that derived from the cells, produced active immunity in mice. Although Perlzweig and his coworkers regarded the immunizing material as protein in character they pointed out evidence suggestive of its non-protein nature.

Schiemann and his collaborators (6, 7) (1927, 1931) first brought convincing evidence that the type-specific polysaccharide of Pneumococcus Type I, in the form isolated by them, produced specific immunity when injected in relatively minute amounts into mice. They also found that if administered to mice in larger doses, this form of the specific carbohydrate not only failed to incite immunity but on the contrary was often toxic and caused purpura. Wadsworth and Brown (11, 12) (1931, 1933) isolated from the bacterial cells a specific fraction designated by them "the cellular carbohydrate." This substance corresponded in its antigenic and purpura-producing action to the carbohydrate of Schiemann and Casper (6), and was similar in its immunological reactions to the A substance of Enders (8). Felton (14) (1932) isolated from Pneumococcus Type I a substance inducing type-specific immunity in mice which from its chemical properties he concluded was "a non-polysaccharide and probably non-protein derivative" of the bacterial cells.

The consistently negative results of all former attempts in this laboratory to induce active immunity in mice with the specific carbohydrate are now known to have been due to the fact that the polysaccharide was then used only in its deacetylated form. As shown in Table V, the change from the antigenic to the non-antigenic form of the carbohydrate is brought about whenever the originally active acetyl polysaccharide is converted by alkali into its deacetylated derivative. This difference in antigenic action, like that already noted in the serological behavior of the two forms of the polysaccharide, is referable to known differences in chemical constitution.

An analysis of the specific reactions of the acetyl polysaccharide discloses a previously unsuspected similarity between this form of the specific carbohydrate and the antigenically active fractions described by other investigators. From the chemical and immunological properties of the acetyl polysaccharide it seems highly probable that this substance in the purified state accounts for the antigenic action of the carbohydrate of Schiemann and Casper (6) and of Wadsworth and Brown (11, 12). As in the case of these substances, the acetyl polysaccharide is antigenically effective in mice only when administered in extremely minute quantities. Although an extensive study of the purpura-producing action of the acetyl polysaccharide has not been made, in several instances purpura has been noted in mice injected with amounts of this substance ranging from 0.4 to 4.0 mg.

That the antigenic action of the water-soluble fraction of Perlzweig and his coworkers (4, 5) may have been due to the presence of traces of unhydrolyzed acetyl polysaccharide seems not unlikely from the readiness with which it lost its immunizing capacity when heated in alkaline solution.

As pointed out earlier, the A substance of Enders (8) and the cellular carbohydrate of Wadsworth and Brown (11, 12) correspond in their serological reactions to those of the acetyl polysaccharide. In addition, both of these substances were shown to be equally sensitive to the destructive action of alkali. While it cannot be stated with certainty that these substances are identical, their properties parallel those of the acetyl polysaccharide so closely that it seems probable that their biological activity is due to this substance.

5. *Antigenic Action of the Acetyl Polysaccharide in Rabbits*

Despite the number of observations on the antigenicity in mice of specific fractions derived from Pneumococcus Type I, comparatively little work has been done to determine the capacity of these substances to incite antibody formation in rabbits. The following experiments, therefore, were carried out to ascertain whether the acetyl polysaccharide possesses the property of stimulating the production of type-specific antibodies in rabbits.

Six rabbits were given intravenous injections of acetyl polysaccharide daily for 6 days, followed by a rest period of 1 week. Three courses of injections were given in all. Two rabbits received 1 cc. of 1:1000 solution of the substance; two others were injected with 1 cc. of 1:10,000 solution; the remaining two were given 1 cc. of 1:100,000 solution. At the end of the third course of injections, each of the three groups of rabbits had received a total quantity of acetyl polysaccharide amounting to 18 mg., 1.8 mg. and 0.18 mg. respectively. 7 days after the second and third series of injections, test bleedings were made and the sera were tested for the presence of type-specific agglutinins, precipitins and protective antibodies.

The detailed protocols of the serological tests are omitted, since in no instance were type-specific agglutinins, precipitins or protective antibodies demonstrable in the serum of the rabbits which had previously received repeated injections of Type I acetyl polysaccharide. Ten days after the last course of injections, each rabbit was infected by the intradermal injection of 0.2 cc. of undiluted blood broth culture of Pneumococcus Type I according to the method described by Goodner (21). The infected rabbits developed at the site of inoculation typical lesions characterized by areas of massive edema and hemorrhagic necrosis. All six of the animals died within 48 to 96 hours after the onset of infection. In no instance, therefore, was there any evidence of increased resistance brought about by the prolonged series of injections of Type I acetyl polysaccharide in amounts totaling 0.18 to 18 mg. In view of the fact that only minute amounts of the acetyl polysaccharide were effective in evoking an immune response in mice, it is conceivable that these rabbits were given too large doses; however, in terms of body weight, the dosage in those rabbits that had received a total of 0.18 mg. was presumably within the range of the amounts found to be effective in mice.

In view of these results, it is significant that the presence of the acetyl polysaccharide was demonstrated by the precipitin reaction in the serum of these rabbits 7 days after the second and third course of injections. This observation indicates that the acetyl polysaccharide is only very slowly excreted, and indirectly suggests that no antibodies were formed, otherwise the substance would in all probability have disappeared more rapidly from the circulation. That the acetyl polysaccharide is actually excreted as such by the kidney and appears in the urine in this specifically reactive form was shown in the case of two other rabbits. One of these animals was given a single large dose of 17 mg. of active substance intravenously and the other a similar amount intraperitoneally. Samples of urine from these animals were collected and tested for the presence of the acetyl polysaccharide. Specific precipitation occurred in the urine of both rabbits on the addition of Type I antipneumococcus serum from which all antibodies reactive with the deacetylated polysaccharide had been previously removed by specific absorption. The specificity of this test conclusively demonstrates that the polysaccharide was excreted in the acetylated form. The urine of both rabbits still showed the presence of the acetyl polysaccharide, as demonstrated by the specific precipitin reaction, 7 days after injection, at which time the observations were discontinued.

Under the conditions of this experiment, the acetyl polysaccharide failed to induce any immune response in rabbits. The serum of the treated animals contained no demonstrable antibodies, and the animals themselves were not protected against subsequent infection with organisms of the homologous type. Moreover, it has been shown that the acetyl polysaccharide persisted in the circulation of the treated rabbits for considerable periods of time, was slowly excreted by the kidney and appeared in the urine in its naturally acetylated form.

It is of course possible that in the present instance the failure of the acetyl polysaccharide to induce antibody formation or to incite active immunity may be attributed to the inadequate number of rabbits tested or to the use of improper amounts of the substance. No assumption is made as to the difference in the antigenic action of the acetyl polysaccharide in mice and in rabbits. The explanation must await further study of this interesting and significant phase of the problem.

DISCUSSION

So far as is known, the only chemical difference between the acetyl polysaccharide and its deacetylated derivative lies in the presence or absence of the acetyl groups. Evidence in support of this view is the difference observed in the acid equivalents of the two forms of the specific carbohydrate. On alkaline hydrolysis there is liberated from the acetyl polysaccharide approximately 6 per cent of acetic acid which is organically bound in the intact molecule in the form of an acetyl ester. From solutions of the acetyl polysaccharide that have been treated with alkali, the deacetylated carbohydrate has been recovered and the substance thus derived has been found to correspond in chemical and serological properties to the polysaccharide formerly known as the "soluble specific substance."

The acetyl polysaccharide possesses all the specific immunological characteristics of the deacetylated derivative and in addition exhibits other distinctive properties. In highly purified form the acetyl polysaccharide, in contradistinction to the deacetylated substance, completely absorbs all demonstrable type-specific antibodies from antiserum of the homologous type; it induces active immunity and incites purpura in mice; it is specifically precipitable in immune serum from which the type-specific anticarbohydrate precipitins reactive with the deacetylated polysaccharide have been removed by specific absorption; it is extremely unstable to the action of alkali.

The results of the present study offer an explanation of many of the perplexing problems that have arisen concerning the nature and specific properties of the soluble specific substance. One of these is the question of the antigenicity of the specific carbohydrate. In the form in which it was originally isolated the polysaccharide was found to be devoid of antigenic action in mice and in rabbits, and considerable evidence was presented that this substance functioned only as a hapten. However, a number of investigators (4, 5, 14, 20) have isolated substances, in some instances of undoubted carbohydrate nature (6, 12), which were antigenic, inducing type-specific immunity in mice. The present experiments show that minute quantities of the purified acetyl polysaccharide give rise to active immunity in mice. While it is impossible to state that the antigenic activity of the specific fractions isolated by others is due to the presence of the acetyl polysaccharide in the preparations employed, this possibility seems not unlikely. The differences between the antigenic and non-antigenic forms of the specific carbohydrate are thus related to known differences in chemical constitution. The antigenicity of the acetyl polysaccharide, in mice at least, is intimately associated with the presence of the acetyl groups in the polysaccharide molecule.

The writers have never maintained that complex carbohydrates may not function as antigens, but until the present experiments with the highly purified acetyl polysaccharide they had obtained no evidence in experimental animals that this was the case. Many years ago, Ulenhuth (22) (1905) presented evidence of the antigenic action of gum arabic, pointing out that this was the first time that specific antibodies had been demonstrated in the serum of animals immunized with a carbohydrate. Recently in collaboration with Remy, Uhlenhuth (23) (1933) has confirmed his early observations, showing that after prolonged immunization with gum arabic, the serum of the treated rabbits contained specific precipitins and complement-fixing antibodies. Ford (24, 25) (1906–07) found that the serum of rabbits immunized with extracts of *Amanita phalloides* and *Rhus toxicodendron*, possessed marked antihemolytic and antitoxic properties. The active principle of these extracts was isolated and identified in each instance as a glucoside.

A question that has been difficult of interpretation is that relating to the purpura-producing activity of the specific carbohydrate. Specific substances of carbohydrate nature isolated by other workers have been found to incite purpura in mice, while the polysaccharide in the form originally isolated does not possess this activity. However, the fact that the mere presence of acetyl groups in a physiologically active substance may greatly modify its activity is well known in the case of acetyl choline which has at least one thousand times the activity of the parent base (26). It is not certain that the purpura-producing activity of the acetyl polysaccharide is solely related to the presence of these groups in the molecule. However, it is known that with loss of acetyl groups, the polysaccharide also loses the capacity to induce purpura.

Another of the perplexing problems that have arisen has been the possibility, indicated by the work of several investigators, that the specific carbohydrate is not the only substance concerned in the type specificity of Pneumococcus Type I. The concept of two type-specific substances has its origin in the observation that the polysaccharide in the form first isolated does not absorb all the type-specific antibodies from immune serum (10). This fact suggested the presence in the serum of antibodies distinct from the anticarbohydrate precipitins, and the coexistence in the cell of another substance unrelated to the polysaccharide. Support for this view was found in the demonstration and subsequent isolation by others (8, 12) of a substance that was specifically precipitable in antiserum from which all precipitins for the specific carbohydrate had been removed. Several explanations were proposed, chiefly that there exist in the cell two specific substances giving rise to two distinct antibodies, both type-specific, but each reactive only with the corresponding antigen. On the other hand, in support of the view that only a single substance is responsible for type specificity Wadsworth and Brown (11) suggested that the specific carbohydrate as first isolated may be only a radical of a more complex substance. Ward (13) suggested "the possibility that the reacting substance in the autolysate is more complex and less stable than the carbohydrate—perhaps a substance intermediate between the antigenic carbohydrate compound in the intact pneumococcus and the carbohydrate itself."

From the work of Landsteiner and others it is known that the mere presence of relatively small chemical groups in an immunologically active substance exerts a determining influence on its specificity. The present study brings evidence that in Pneumococcus Type I the specific carbohydrate with the acetyl groups intact fulfils all the serological requirements of type specificity. On the other hand, when the acetyl groups are removed the resultant product retains the polysaccharide structure and the dominant type specificity of the original carbohydrate, but loses many of the specific characteristics that distinguish the naturally acetylated polysaccharide.

SUMMARY

The soluble specific substance of Pneumococcus Type I has been chemically isolated from the bacterial cells and from autolyzed cultures as an acetyl polysaccharide.

So far as could be determined by the methods employed, the acetyl polysaccharide in highly purified form absorbs from Type I antipneumococcus serum all demonstrable type-specific precipitins, agglutinins and protective antibodies.

Mice injected intraperitoneally with minute quantities of the acetyl polysaccharide develop active immunity to subsequent infection with Pneumococcus Type I. The immunity thus induced is type-specific. In several instances purpura has been observed in mice following the injection of larger amounts of the acetyl polysaccharide.

Under the experimental conditions of this study, no type-specific precipitins, agglutinins or protective antibodies were demonstrable

in the serum of rabbits following repeated intravenous injections of the Type I acetyl polysaccharide. The treated rabbits were not immune to subsequent infection with Pneumococcus Type I.

The acetyl polysaccharide is readily converted into its deacetylated derivative by treatment with dilute alkali.

The chemical and immunological properties of the deacetylated polysaccharide are identical with those of the soluble specific substance in the chemical form in which it was originally isolated; the deacetylated form of the specific carbohydrate is non-antigenic, does not produce purpura in mice, and only incompletely absorbs the type-specific antibodies from Type I antipneumococcus serum.

The immunological significance of the acetyl polysaccharide and its possible relationship to the specific substances isolated from Pneumococcus Type I by other workers are discussed.

CONCLUSIONS

The soluble specific substance of Pneumococcus Type I is now regarded, tentatively at least, as an acetyl polysaccharide. In this form it accounts adequately for all the serological phenomena of type specificity of Pneumococcus Type I.

Addendum.—During the course of publication of the present work there has appeared a paper by Pappenheimer and Enders[4] on the specific carbohydrate of Type I Pneumococcus. On the basis of elementary analysis, amino nitrogen content and specific rotation, these authors conclude that the A substance of Enders and the specific polysaccharide previously isolated in this laboratory are closely related and that the latter is possibly a hydrolytic product of the former substance.

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